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Award Number: DAMD17-98-1-8345

TITLE: Effect of Folate on the Efficacy and Toxicity of Cancer  
Chemotherapy

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REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;  
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# REPORT DOCUMENTATION PAGE

OMB No. 074-0188

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<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> September 1999	<b>3. REPORT TYPE AND DATES COVERED</b> Annual (1 Sep 98 - 31 Aug 99)	
<b>4. TITLE AND SUBTITLE</b> Effect of Folate on the Efficacy and Toxicity of Cancer Chemotherapy			<b>5. FUNDING NUMBERS</b> DAMD17-98-1-8345	
<b>6. AUTHOR(S)</b> Richard F. Branda, M.D. Dr. John McCormack				
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<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b>  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>  This report contains colored photos				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for public release; distribution unlimited				<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b>  The purpose of this research project is to understand better the effect of dietary folate levels on the cellular pharmacology and toxicology of chemotherapeutic agents. The scope of the research involves <i>in vitro</i> studies with cell lines and <i>in vivo</i> assessments in rats of folate-chemotherapeutic drug interactions. Studies at a molecular level led to a new model to explain the synergy between nutritional folate deficiency and alkylating agents. Our studies at a cellular level suggest that folate metabolism can modulate glutathione levels. This observation may explain at least in part why dietary folate levels influence the efficacy and toxicity of alkylating agents. Studies in rats confirm that dietary folate levels affect the toxicity of cyclophosphamide, but there may be an optimal amount of dietary folate to reduce that toxicity. These <i>in vivo</i> studies also indicate that other aspects of diet are important in determining sensitivity to chemotherapy. Rats maintained on a cereal-based diet were much more resistant to the toxic effects of cyclophosphamide than rats eating a Purified Diet. Taken together, our studies suggest that dietary changes can have a profound and largely unappreciated effect on the outcome of cancer chemotherapy.				
<b>14. SUBJECT TERMS</b> Breast Cancer			<b>15. NUMBER OF PAGES</b> 45	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

NSN 7540-01-280-5500

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*Richard F. Boud* 8/26/89  
PI - Signature Date

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U.S. Army Medical Research and Materiel Command  
Animal Use Report

### Manuscripts

Branda, R.F., Lafayette, A.R., O'Neill, J.P., Nicklas, J.A. The effect of folate deficiency on the *hprt* mutational spectrum in Chinese hamster ovary cells treated with monofunctional alkylating agents. *Mutation Res.* 427:79-87, 1999.

Branda, R.F., Nigels, E., Lafayette, A.R., Hacker, M. Nutritional folate status influences the efficacy and toxicity of chemotherapy in rats. *Blood* 92:2471-2476, 1998.

## INTRODUCTION

The general subject of this research project is the effect of diet and nutrition on the efficacy and toxicity of chemotherapy in women with breast cancer. More specifically, the research focuses on the interactions of a micro-nutrient, folic acid, with chemotherapeutic drugs frequently used clinically in women with breast cancer in either the adjuvant or metastatic setting. The hypothesis to be tested is whether dietary supplementation with a non-toxic nutrient, folic acid, may reduce the toxicity and increase the efficacy of chemotherapy in women with breast cancer. The purpose of the research is to better understand the effect of folate metabolism and varying dietary folate levels on the cellular pharmacology and clinical toxicity of chemotherapeutic agents and then utilize this knowledge to decrease the toxicity and increase the effectiveness of these drugs. The scope of the research involves *in vitro* studies with cell lines to assess folate-drug interactions and *in vivo* assessments in rats of folate-chemotherapeutic drug interactions.

## BODY

### Task 1. *In vitro* assessment of folate-drug interactions

- Determine the impact of folate levels on the cytotoxicity of 5-FU, doxorubicin and hydroperoxycyclophosphamide

The cell lines described in the grant application: MCF-7 (mammary adenocarcinoma); BT-474 (mammary ductal carcinoma); SK Br3 (mammary ductal carcinoma); MDA-MB-435 (mammary adenocarcinoma); and Hs578 Bst (human breast fibroblast cells) have been located in tissue repositories and obtained. They are being grown out in their original media and adapted to growth in media that can be formulated as folate-free. In the meantime, experiments are being performed with MADB106 rat mammary carcinoma cells and with TK6 human lymphoblastoid cells instead of the RPMI 1788 human lymphoblastoid cells described in the grant. We decided that the TK6 cells were preferable because we already have them adapted to growth in media that can be formulated folate-free, and a variant mutated at the p53 gene (WTK1) is available in our laboratory. The latter cells may prove useful to investigate the role of p53 mutations on the interaction of folate metabolism and chemotherapeutic drugs.

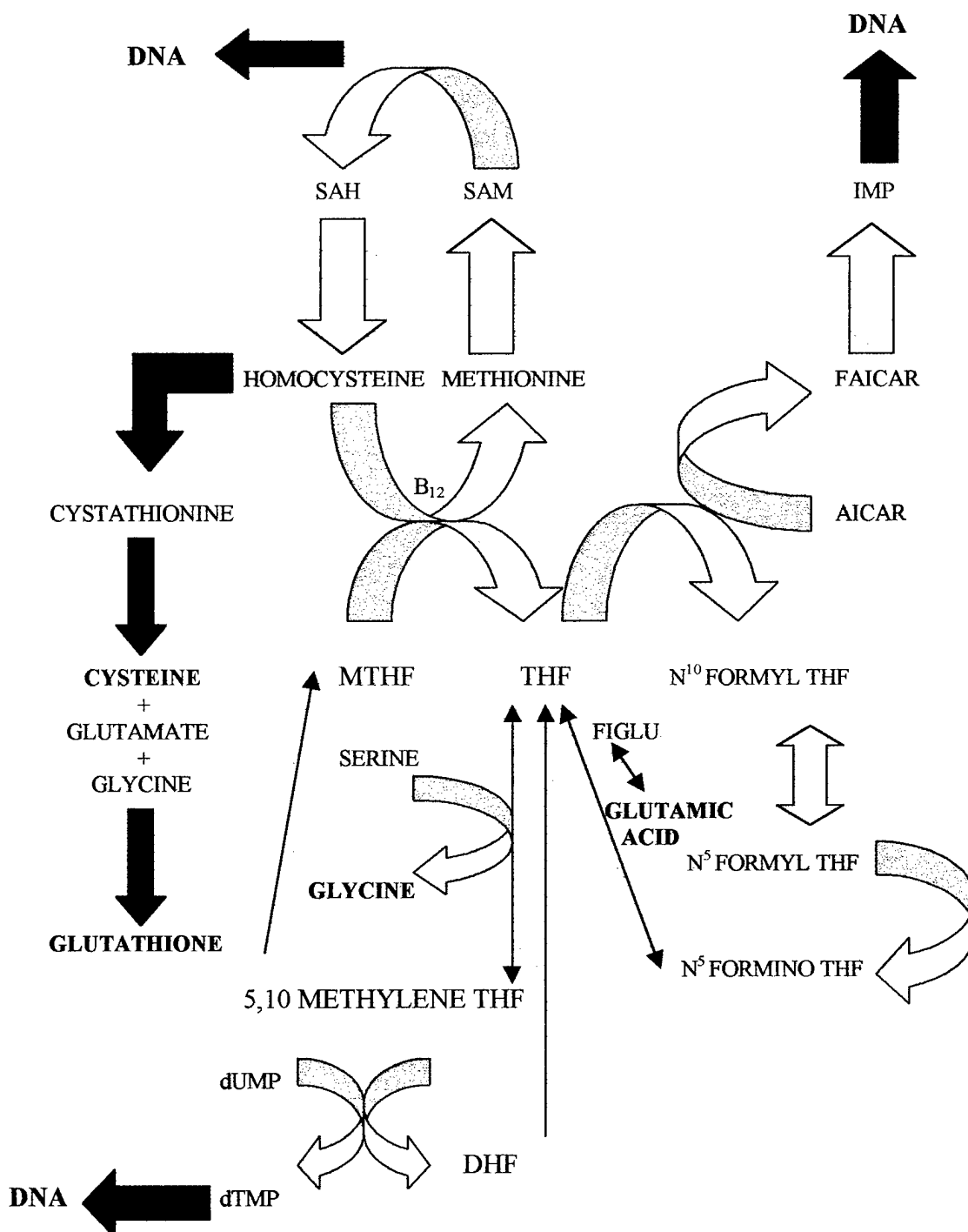
We encountered considerable difficulty identifying a source of hydroperoxycyclophosphamide. It is not commercially available, and most of the currently synthesized drug is committed to studies of bone marrow purging in preparation for bone marrow transplantation. We were able to obtain a small quantity from Dr. Carol Miller at the Johns Hopkins Medical School. Our plan is to perform the studies described in the grant with 5-FU, methotrexate and doxorubicin, and use ethyl methanesulfonate (EMS) as a surrogate for cyclophosphamide, since its mechanism of action is similar. We will then repeat critical experiments with hydroperoxycyclophosphamide when the experimental conditions have been defined.

- Determine mechanism of folate-drug interactions
  - Assess DNA synthesis
  - Perform alkaline/neutral elutions to determine DNA lesions
  - Western blots to determine topoisomerase II/PGP expression where appropriate (budget support not approved)
  - Enzymatic/drug uptake studies for protein function

During the past year we studied the mechanism of folate-drug interactions at the molecular and cellular levels. The results of the molecular studies and the methods used were recently published in *Mutation Research* and the manuscript is appended (1). Briefly, we found that folic acid deficiency acts synergistically with alkylating agents to increase DNA strand breaks and mutant frequency at the *hprt* locus in Chinese hamster ovary (CHO) cells. To elucidate the mechanism of this synergy, molecular analyses of *hprt* mutants were performed. Previously our laboratory showed that folate deficiency increased the percentage of clones with intragenic deletions after exposure to ethyl methanesulfonate (EMS) but not *N*-nitroso-*N*-ethylurea (ENU) compared to clones recovered from folate replete medium. This report describes molecular analyses of the 37 *hprt* mutant clones obtained that did not contain deletions. Folate deficient cells treated with EMS had a high frequency of G>A transitions at non-CpG sites on the non-transcribed strand, particularly when these bases were flanked on both sides by G:C base pairs. Thirty-three percent of these mutations were in the run of six G's in exon 3. EMS-treated folate replete cells had a somewhat lower percentage of G>A transitions, but the same sequence specificity. Treatment of folate deficient CHO cells with ENU resulted in predominantly T>A transversions and C>T transitions on the non-transcribed strand. All mutations induced by either EMS or ENU at G bases occurred preferentially in the non-transcribed strand; for example, of 18 G:C to A:T mutations, the G was on the non-transcribed strand 12 times. These findings suggest a model to explain the synergy between folate deficiency and alkylating agents: 1) folate deficiency causes extensive uracil incorporation into DNA; 2) greatly increased utilization of base excision repair to remove uracil and to correct alkylator damage leads to error-prone DNA repair. In the case of EMS, this results in more intragenic deletions and G:C to A:T mutations due to impaired ligation of single-strand breaks generated during base excision repair and a decreased capacity to remove *O*<sup>6</sup>-ethylguanine. In the case of ENU additional T>A transversions and C>T transitions are seen, perhaps due to mis-pairing of *O*<sup>2</sup>-ethylpyrimidines. Correction of folate deficiency may reduce the frequency of these types of genetic damage during alkylator therapy.

In the past year our studies of the cellular pharmacology of the interaction of folate metabolism and chemotherapeutic drugs have focused on the role of folate metabolism in glutathione (GSH) synthesis. As shown in Figure 1, below, folate metabolism is complex, and folate compounds contribute to: 1) the synthesis of nucleotides important for nucleic acid synthesis; 2) methylation reactions by formation of S-adenosyl-L-methionine; and 3) amino acid synthesis. Since folate metabolism is involved in the synthesis of the three amino acids that form GSH (cysteine, glutamic acid, and glycine), we postulated that changes in folate metabolism might alter cellular GSH levels.

Figure 1. Relationship between folate metabolism and glutathione synthesis.



*MTHF*, 5-methyltetrahydrofolate; *B<sub>12</sub>*, vitamin B<sub>12</sub>; *SAM*, S-adenosyl-L-methionine; *SAH*, S-adenosyl-L-homocysteine; *THF*, tetrahydrofolate; *dUMP*, deoxyuridylic acid; *dTMP*, thymidylic acid; *DHF*, dihydrofolate; *FIGLU*, formino-glutamic acid; *AICAR*, 5-amino-4-imidazolecarboxamide; *FAICAR*, formyl-AICAR; *IMP*, inosinic acid.

When given *in vivo*, cyclophosphamide is metabolized, through hydroxylation by the hepatic mixed-function oxidases, to 4-hydroperoxycyclophosphamide. The latter compound exists in equilibrium with its ring-opened tautomer aldophosphamide. GSH reacts with aldophosphamide and with the toxic metabolites of 4-hydroperoxycyclophosphamide, phosphoramidate mustard and acrolein (2,3). As a result, administration of cyclophosphamide *in vivo* depletes cellular GSH levels. On the other hand, cellular GSH levels influence the toxicity and efficacy of cyclophosphamide. Thus, tumor cell lines that manifested a 40% or greater depletion of GSH after treatment with 4-hydroperoxycyclophosphamide had marked reduction of clonogenic cell survival, while cells that maintained GSH levels were resistant to 4-hydroperoxycyclophosphamide (2). Therefore, an increased level of GSH in tumor cells as a result of increased intracellular GSH synthesis protects the cells against the cytotoxicity of alkylating agents such as cyclophosphamide (3). Conversely, experimental depletion of GSH increases the sensitivity of animals and/or cells to the toxic effects of cyclophosphamide (4). Since cysteine is the usual limiting substrate in the synthesis of GSH (4), and folate metabolism influences cysteine (as well as glutamate and glycine synthesis) (Figure 1) we have studied the effect of low dietary folate levels on GSH levels. Our preliminary results are shown in Figures 2 and 3.

Free reduced glutathione was analyzed by HPLC with fluorescent detection following pre-column derivatization with *o*-phthalaldehyde (OPA) by the method of Paroni et al (5) with a slight modification in the HPLC elution method. The HPLC conditions consisted of using either a Waters Nova-Pak (3.9 x 150 mm) C-18 column or a Phenomenex Luna (4.6 x 150 mm) C-18 column, both with a Phenomenex guard column. The mobile phase consisted of 150 mM (pH 7.0) sodium acetate buffer with variable (0 and 5%) quantities of acetonitrile (v/v). After each analysis (12 min run time), the column was washed with 100% acetonitrile (5 min) and equilibrated (6 min) with the mobile phase. The retention time of glutathione was approximately 7 minutes and glutathione was cleanly resolved from all other peaks. For cultured cells (approx. 1 million cells/pellet), the cell pellet was sonicated with 75  $\mu$ L of 0.29 N perchloric acid (to precipitate proteins) on ice, centrifuged (5 min at 10,000g) and the supernatant was analyzed by HPLC. For liver tissue, a small tissue sample (approx. 20-30 mg) was cut (on dry-ice) from the frozen intact livers and placed into tared tubes. The samples were stored at  $-80^{\circ}\text{C}$  until analysis, then weighed and sonicated with perchloric acid at a ratio of 10 mg tissue per 100  $\mu$ L acid, centrifuged (5 min at 10,000g), diluted 1 to 10 in perchloric acid and then analyzed by HPLC. External standards were used for quantitation and the standard curve was linear from 100  $\mu$ M to 1 nM.

Figure 2 shows GSH levels in MADB106 rat mammary tumor cells and TK6 human lymphoblastoid cells after growth in culture medium lacking folic acid (Low Folate) or in culture medium containing adequate amounts of folic acid (Folate Replete). After 1 and 2 days in culture, during a period of rapid growth, the cells from both lines in folate-deficient medium have lower GSH levels than control cells in complete medium. In quadruplicate determinations, these differences are significant at  $P>0.01$ . When growth slows on days 3 and 4 of culture, these differences are no longer significant.



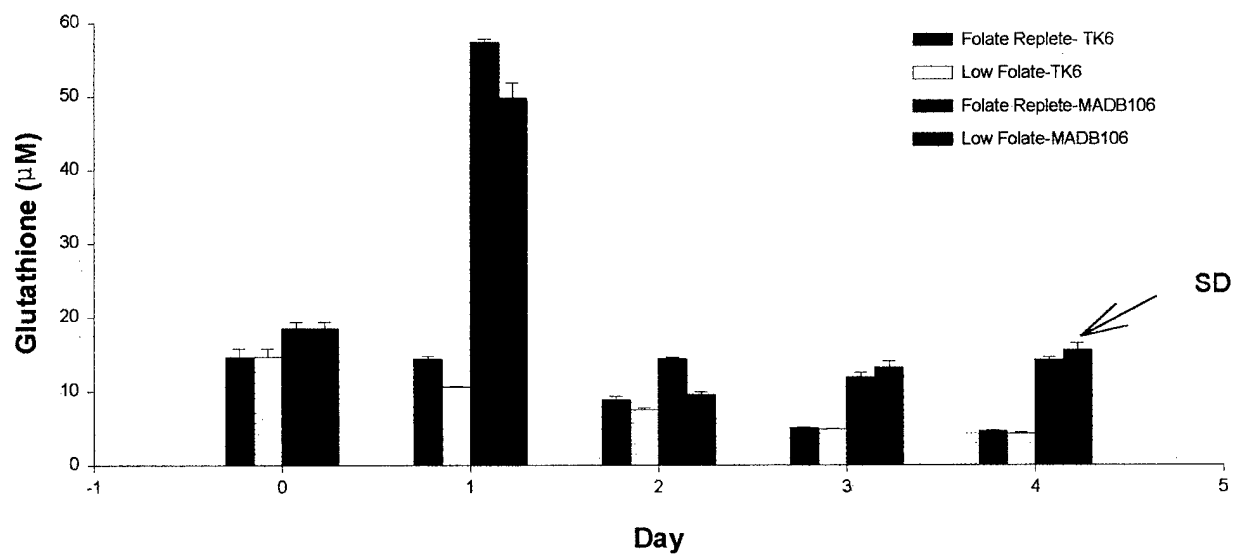


Figure 2. Glutathione levels in cells grown in media of varying folate content

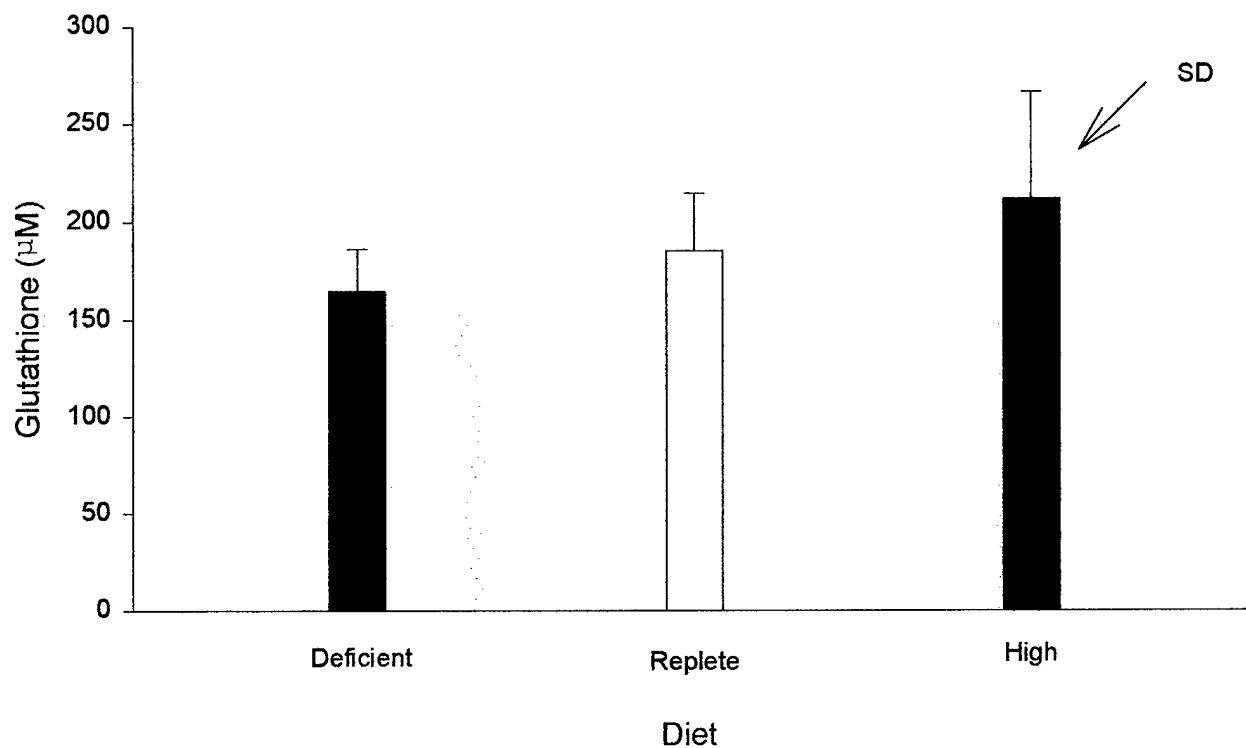


Figure 3. Effect of diets of varying folate content on rat hepatic glutathione levels

Figure 3 shows the results of analyses of liver GSH levels in rats maintained on diets of varying folate content. These livers were collected from rats that had been treated with cyclophosphamide, 50 mg/kg, after 5 weeks on the diets and 2 weeks prior to sacrifice, as described in the experiments below. Supporting our findings in Figure 2 with cell lines, rats on the folate deficient diets had lower GSH levels than rats on folate replete diets, while rats supplemented with high levels of folate had increased GSH levels. Therefore the hepatic GSH levels were proportional to the dietary folate levels in these rats.

These preliminary studies *in vitro* and *in vivo* will be replicated during the coming year to confirm the results. At this point the results support the notion that folate metabolism influences GSH levels: low dietary levels of the vitamin are associated with low GSH levels, while higher than normal dietary levels lead to increased GSH levels. Since GSH levels are important determinants of both the efficacy and toxicity of alkylating agents (2-4), the modulating effect of dietary folate on the efficacy and toxicity of cyclophosphamide may be mediated, at least in part, by varying cellular GSH levels.

#### Task 2. *In vivo* assessment of folate drug interactions

- Determine the effect of folate status on tumor growth rate
- Determine the effect of folate status on drug efficacy
- Determine the effect of folate status on drug toxicity

These experiments are proceeding as described in the grant application. We elected to start with the toxicity studies, since these experiments are the most time-consuming because of the numerous assays for toxicity. In addition, we plan to use the results for dose-finding for the efficacy studies.

Weaning female Fischer 344 rats were maintained on either standard rat chow (Teklad 7012) or a Purified Diet containing either no folic acid or 2 mg folic acid/kg of diet, as previously reported by our laboratory (6; manuscript appended). Some rats on the folate-containing diet received additional folic acid, 50 mg/kg, intraperitoneally (IP) daily. After 5 weeks on these diets, the rats were injected with a single dose of either cyclophosphamide or 5 fluorouracil (5-FU) IP. Blood was obtained for laboratory determinations prior to the chemotherapy injection, and on days 4, 9 and 14 afterward. Surviving rats were sacrificed on day 14 and the livers collected and frozen.

Measurements of hepatic folate levels by the *Lactobacillus casei* method (6) gave the following results: standard rat chow, 34.0 µg/g; folate replete diet, 32.1 µg/g; folate deficient diet, 9.1 µg/g; high folate animals, 45.8 µg/g. These results indicate that the dietary conditions caused important differences in tissue folate levels.

Table 1 shows the number of deaths in each dietary group (6 animals/group) after treatment with increasing doses of cyclophosphamide. These results have not been analyzed statistically yet because the experiments are still in progress.

Table 1. Effect of diet on cyclophosphamide toxicity in rats with death as an end-point.

Dose of Cyclophosphamide (mg/kg)	Diet			
	Control	Folate Deficient	Folate Replete	High Folate
50	0	0	0	1
65	0	0	0	0
85	0	0	1	0
110	0	3	1	3
144	0	2	2	4
190	1	4	4	6
250	4	6	6	6

The weight changes in the rats on the various diets are shown in Figure 4. During the first 5 weeks growth rates were similar and not statistically different, indicating that the diets are nutritionally adequate to support growth. In weeks 7 and 8, rats that were injected with cyclophosphamide in week 6 at a dose of 65 mg/kg or higher had some loss of weight. This effect was most pronounced at the highest dose levels and in rats on the folate deficient diet.

The results of analyses of bone marrow, liver, kidney and cardiac toxicity at the various cyclophosphamide dose levels are shown in Figures 5-11. In these figures, the diets are: CO, control rat chow (cereal-based); FD, folate deficient; FR, folate replete; HF, high folate. Hematocrit, white blood cell counts, and BUN were measured prior to therapy and 4, 9 and 14 days after chemotherapy. Measurements of LDH, SGPT and CPK were done only on day 14 because larger volumes of blood were required and these volumes could only be obtained when the animals were exsanguinated. For the white blood count, the Coulter Counter (Model ZBI) was used. Analyses of BUN, LDH, SGPT and CPK were performed using Sigma Diagnostics (St. Louis, MO) Procedures No. 66-UV, 500, 505 and 661, respectively.

Statistical analyses found no differences in the various indices of toxicity in blood samples obtained prior to chemotherapy, supporting the conclusion from the growth rates that all of the diets were nutritionally adequate. Statistical analyses of the data at the 110 mg/kg dose (Figure 8) by ANOVA showed significant differences for hematocrit and BUN. Fisher's LSD test showed that the folate deficient group was significantly more anemic ( $P < 0.01$ ) than the other dietary groups, while these groups were not different from each other, and had a higher BUN than the other groups ( $P = 0.07$ ). Further statistical analyses are under way. These preliminary results suggest that the combination of folate deficiency and cyclophosphamide leads to increased bone marrow suppression and renal damage. That the combination of diet and cyclophosphamide has an important effect of bone marrow function is supported by an analysis of rats treated with 144

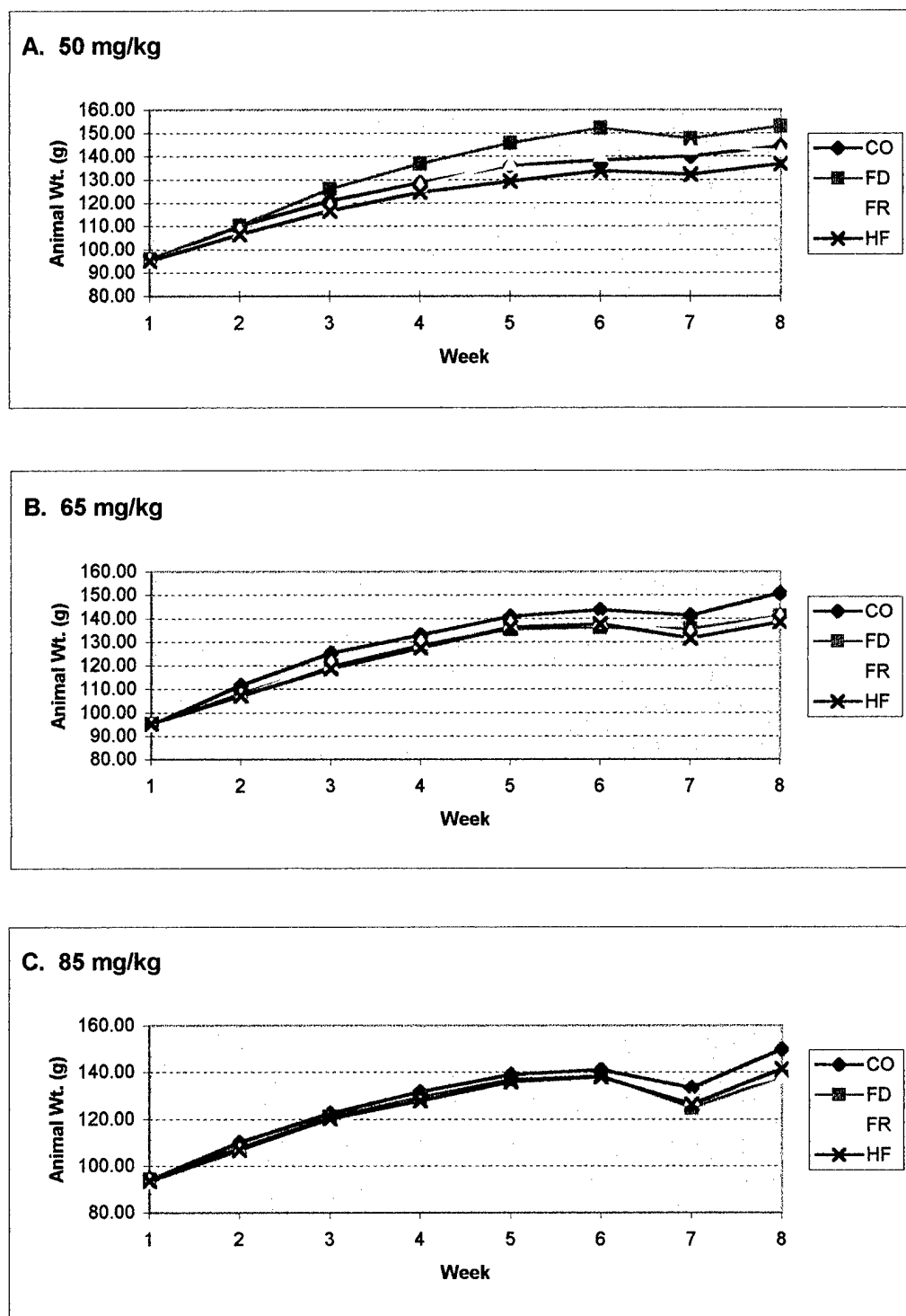
mg/kg. The day 4 white blood cell counts of rats that went on to die by day 14 after treatment were only 70% of those that were alive on day 14 (963/cmm vs 1373/cmm).

Until the experiment is completed, we will not be able to determine an LD<sub>50</sub> using probit analysis. However, inspection of Table 1 suggests that, among animals on the Purified Diet, rats on the folate-replete diet will have the highest LD<sub>50</sub> (i.e. the lowest toxicity) while folate deficient and high folate animals will have greater toxicity. This result is consistent with the idea that nutritional folate status influences alkylating agent toxicity, but suggests that the effect is more complicated than previously recognized. There may be an optimal dietary intake of the vitamin. At present the data indicates that very low or high amounts of dietary folate may increase cyclophosphamide toxicity. It may be of interest that rats on the high folate regimen treated with 144 mg/kg and 190 mg/kg cyclophosphamide, doses at and above the LD<sub>50</sub>, have substantially higher BUNs than the other dietary groups (Figure 9 and 10). Achon and colleagues recently reported that high dietary folate supplementation of rats was associated with impaired dietary protein metabolic utilization and higher urinary nitrogen elimination (7). Fetuses of folate supplemented dams had lower body weight and shorter vertex-coccyx length compared to unsupplemented dams (7).

More surprising, and unexpected, is the remarkable resistance to alkylating agent toxicity shown by the animals on the Teklad 7012 rat chow. This resistance is not mediated by dietary folate levels, because when we measured hepatic folate levels in rats maintained on the Teklad 7012 and folate-replete Purified Diets, they were comparable, as noted above. A comparison of the two diets indicates that they are similar in macro-nutrient content. Total energy, total protein, fat and fiber composition were comparable. There were many differences in micro-nutrient content, and any of these alone or in combination may be important in conferring resistance to alkylating agents in the Teklad diet. Perhaps the most provocative difference between the diets was in antioxidant content. The Teklad 7012 diet contains substantially more vitamin E and vitamin A than the Purified Diet. Others have reported that rats fed methyl/folate-deficient diets had decreased levels of vitamins E, A and C and a compromised antioxidant defense system (8). Alkylating agents such as cyclophosphamide create reactive oxygen species that are more toxic in the absence of antioxidant compounds (9). We showed in Figures 2 and 3 that folate deficiency is associated with lower GSH levels. Our data at present suggests that part of the increased toxicity seen in folate deficient animals may be related to enhanced levels of reactive oxygen species produced by alkylating agents. It will be of interest to see if animals on the Tekad 7012 diet have reduced tumor efficacy of alkylating agents compared to animals on the Purified Diets. Possible mechanisms for reduced toxicity and efficacy would include increased quenching of reactive oxygen species or the enhanced induction of the cytochromes P-450 drug/toxin metabolizing system and mixed function oxidases seen in animals fed cereal-based diets such as Tekad 7012 (10).

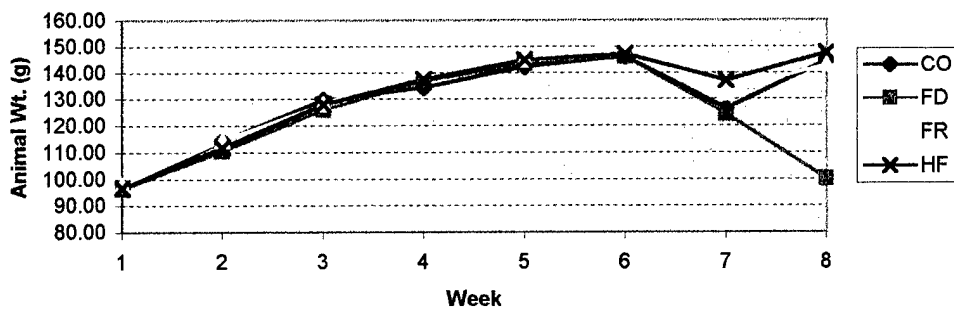
Toxicity studies are also underway with 5 FU. We have started with a dose of 144 mg/kg, which resulted in one death each in the control diet, folate deficient and folate replete diet groups, and 3 deaths in the high folate group. Dose escalation is planned to determine the LD<sub>50</sub> values.

Figure 4. Weight Changes in Rats on Diets of Differing Folate Content Treated in Week 6 with Cyclophosphamide.

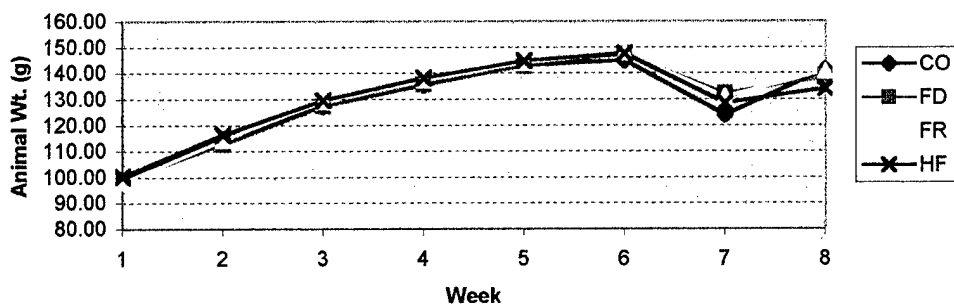


CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

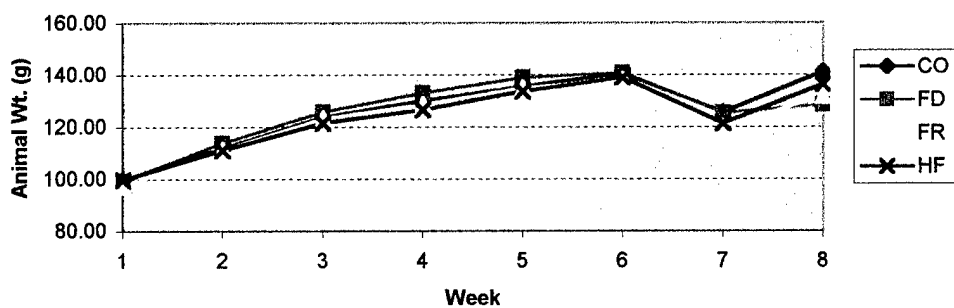
**D. 110 mg/kg**



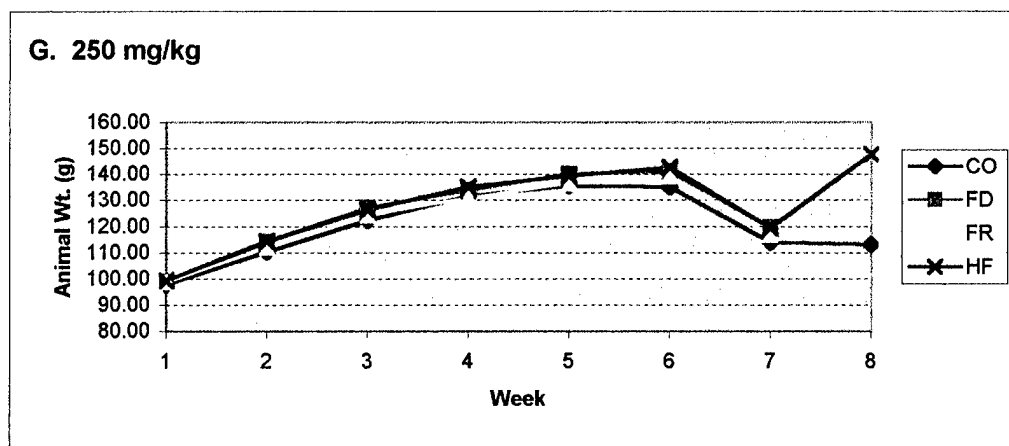
**E. 144 mg/kg**



**F. 190 mg/kg**

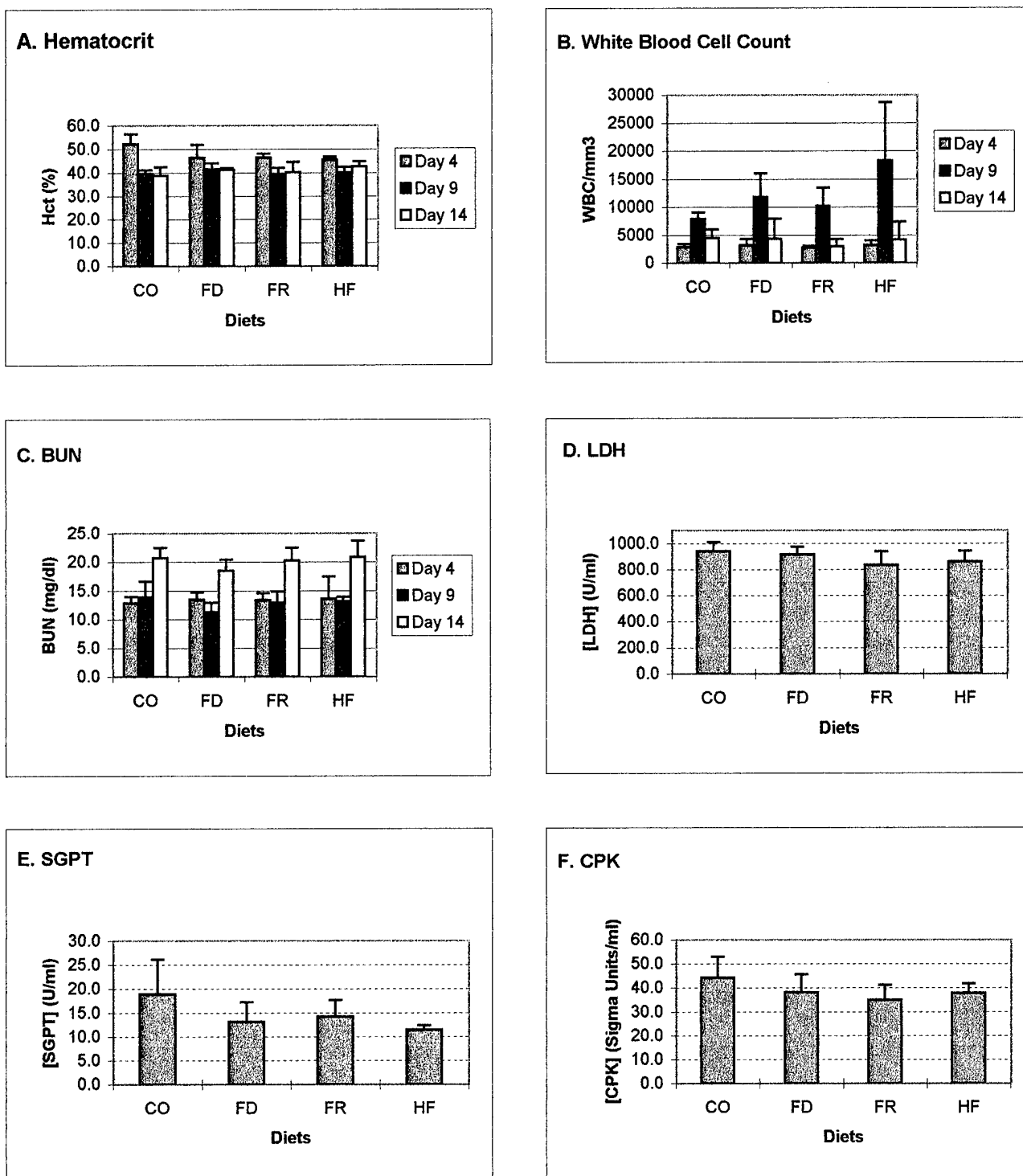


CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet



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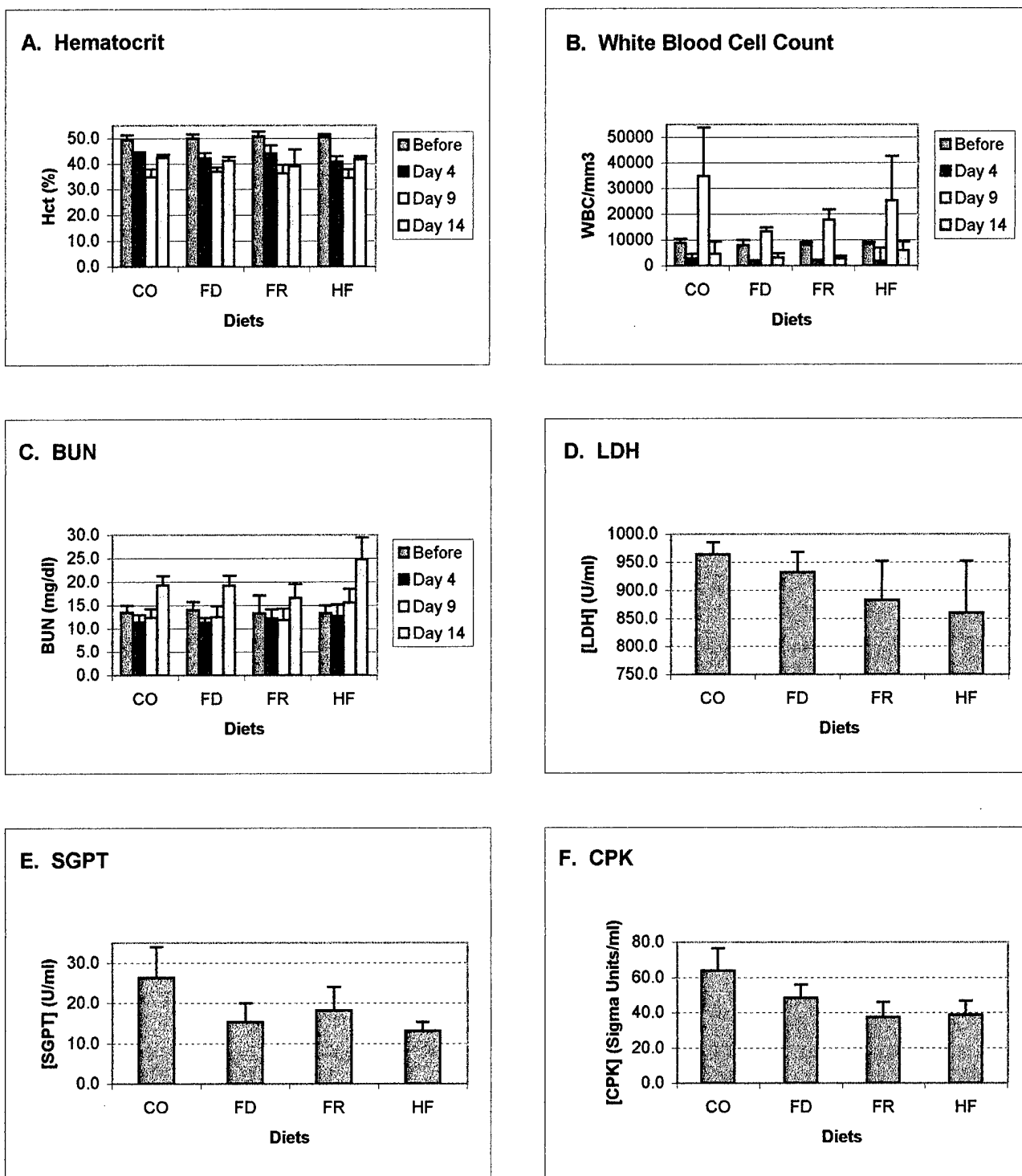
Figure 5. Bone Marrow, Liver, Kidney and Cardiac Toxicity in Rats Measured Before and 4, 9 and 14 Days after Treatment with Cyclophosphamide, 50 mg/kg.



CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

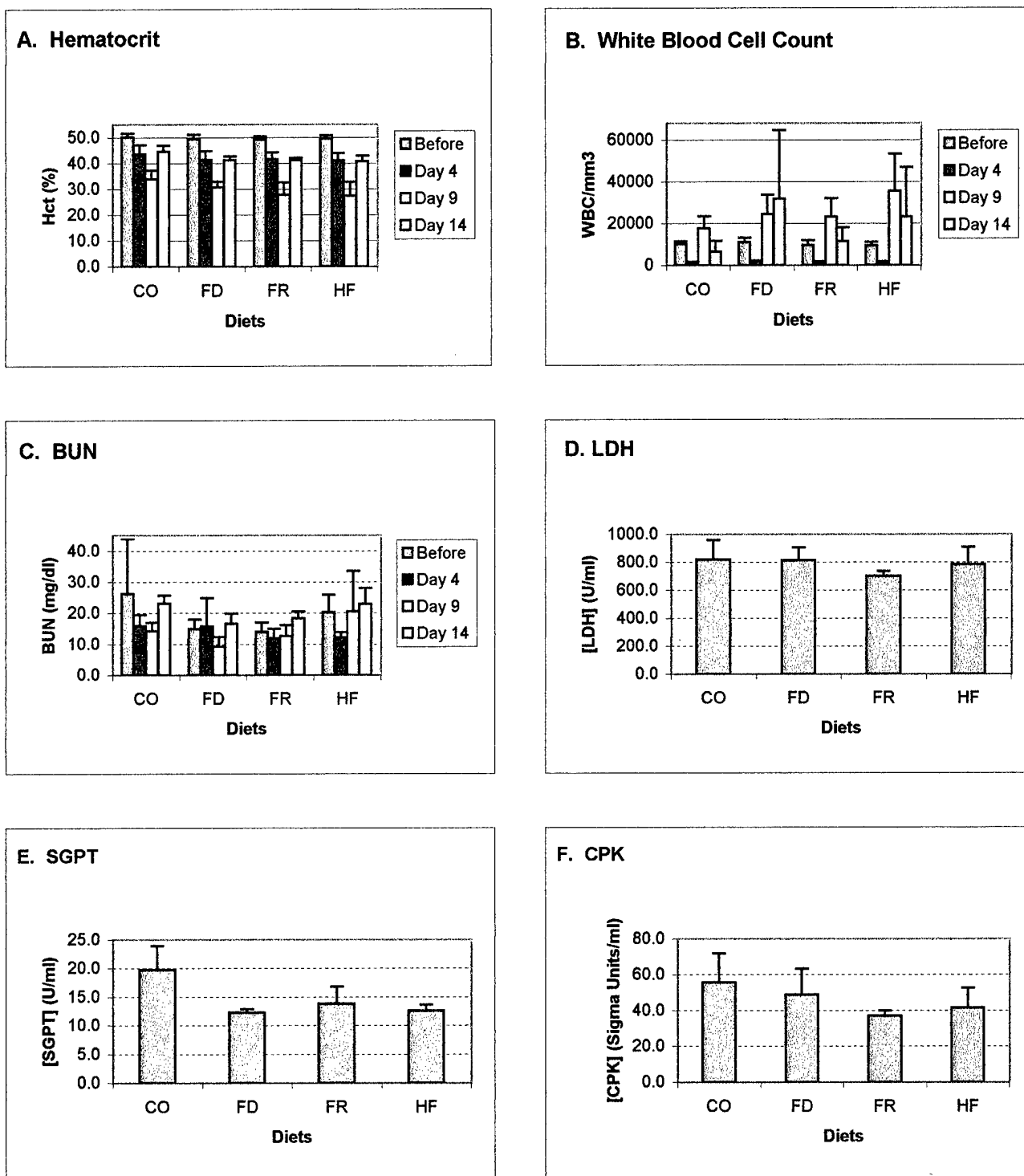


Figure 6. Bone Marrow, Liver, Kidney and Cardiac Toxicity in Rats Measured Before and 4, 9 and 14 Days after Treatment with Cyclophosphamide, 65 mg/kg.



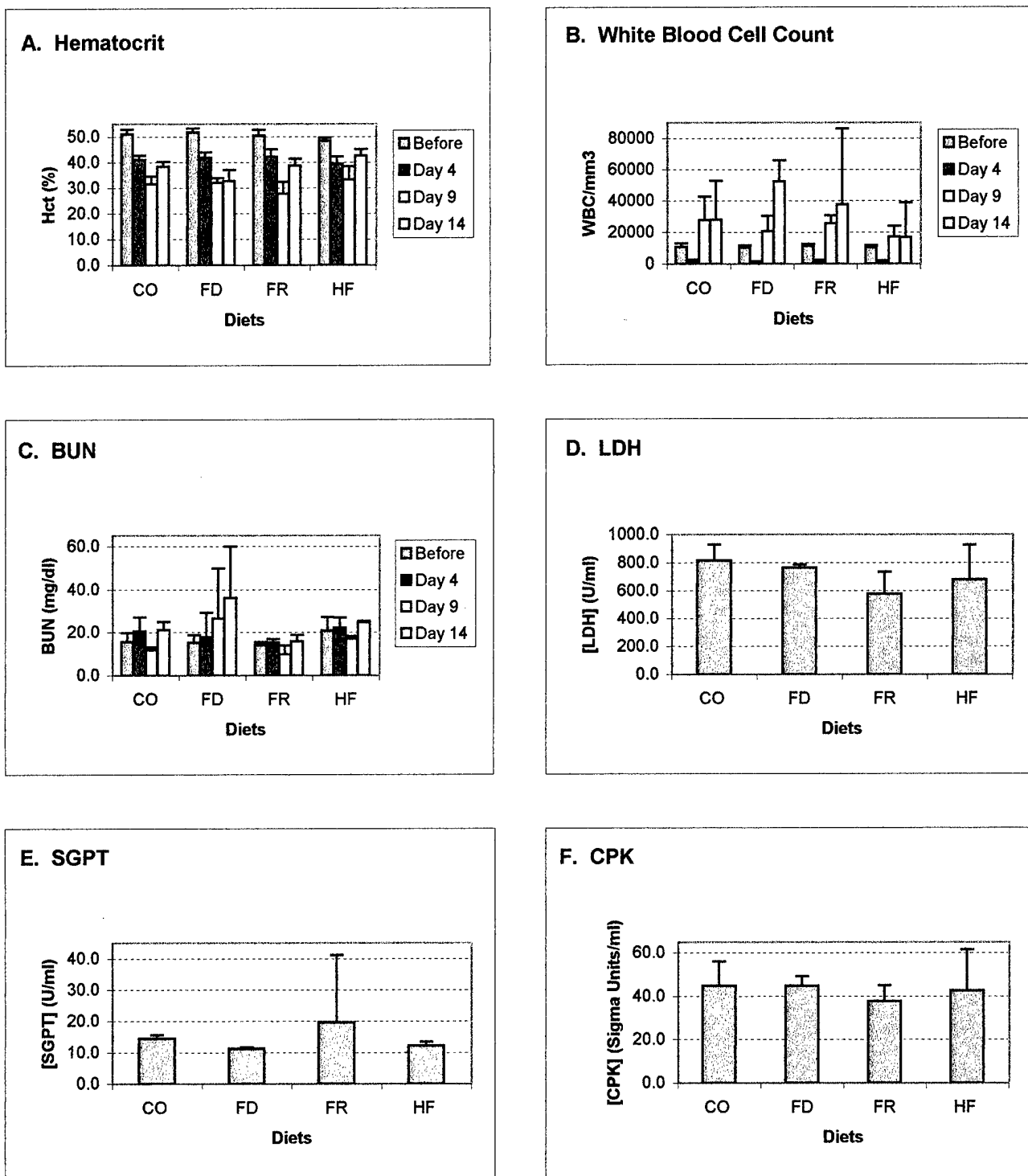
CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

Figure 7. Bone Marrow, Liver, Kidney and Cardiac Toxicity in Rats Measured Before and 4, 9 and 14 Days after Treatment with Cyclophosphamide, 85 mg/kg.



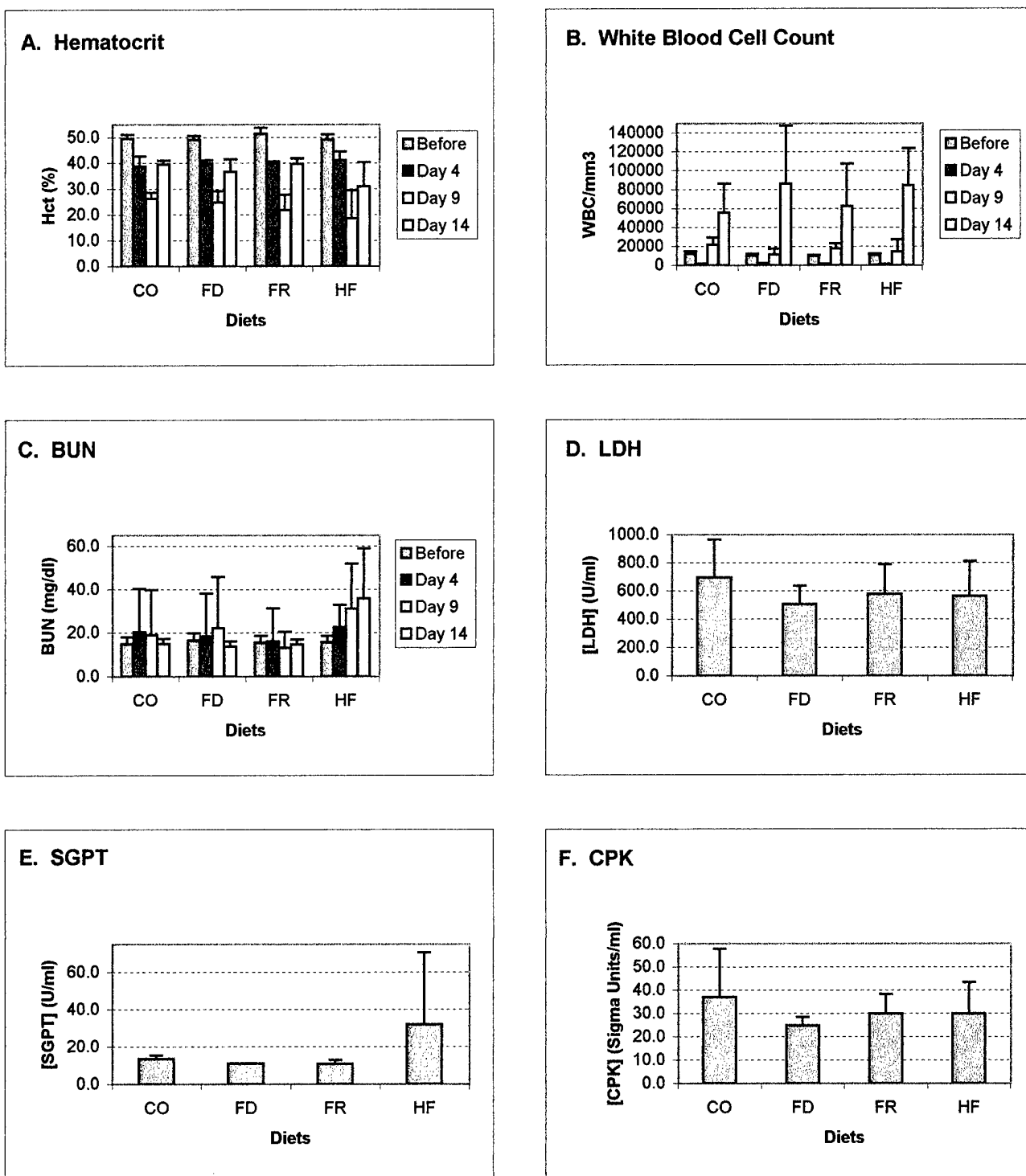
CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

Figure 8. Bone Marrow, Liver, Kidney and Cardiac Toxicity in Rats Measured Before and 4, 9 and 14 Days after Treatment with Cyclophosphamide, 110 mg/kg.



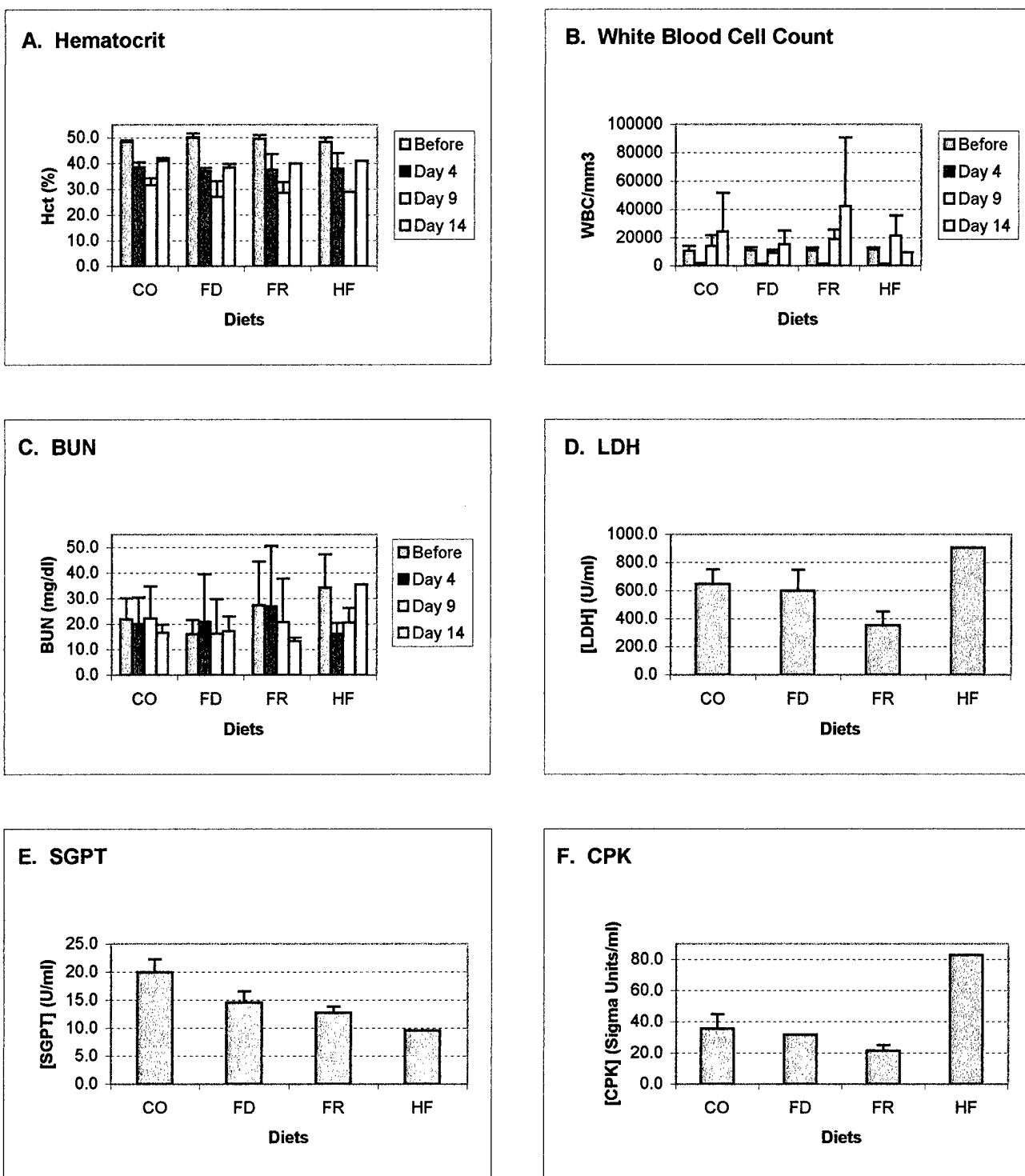
CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

Figure 9. Bone Marrow, Liver, Kidney and Cardiac Toxicity in Rats Measured Before and 4, 9 and 14 Days after Treatment with Cyclophosphamide, 144 mg/kg.



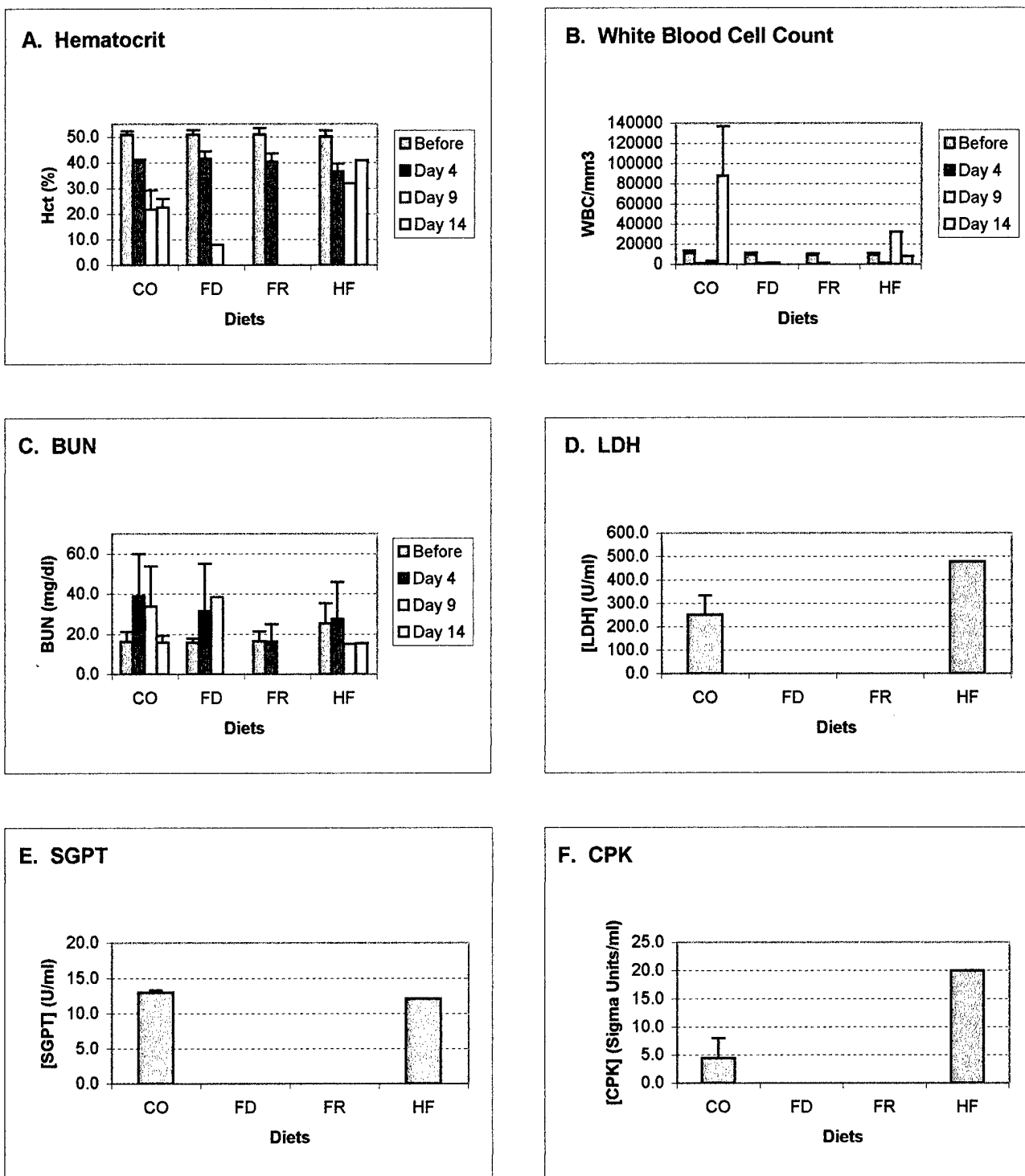
CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

Figure 10. Bone Marrow, Liver, Kidney and Cardiac Toxicity in Rats Measured Before and 4, 9 and 14 Days after Treatment with Cyclophosphamide, 190 mg/kg.



CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

Figure 11. Bone Marrow, Liver, Kidney and Cardiac Toxicity in Rats Measured Before and 4, 9 and 14 Days after Treatment with Cyclophosphamide, 250 mg/kg.



CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

## KEY RESEARCH ACCOMPLISHMENTS

- Studies of the interaction of folate metabolism and chemotherapeutic drugs at the molecular level led to a new model to explain the synergy between nutritional folate deficiency and alkylating agents.
- A previously unrecognized relationship between folate metabolism and cellular glutathione levels was found *in vitro* in cell lines and *in vivo* in rat liver.
- In on-going experiments, dietary folate levels influence the toxicity of an alkylating agent, cyclophosphamide, in rats.
- Rats on a cereal-based diet were remarkably more resistant to the toxicity of cyclophosphamide than rats on a Purified Diet.

## REPORTABLE OUTCOMES

Manuscript: Branda, R.F., Lafayette, A.R., O'Neill, J.P., Nicklas, J.A. The effect of folate deficiency on the *hprt* mutational spectrum in Chinese hamster ovary cells treated with monofunctional alkylating agents. *Mutation Res.* 427:79-87, 1999.

## CONCLUSIONS

Dietary supplements have become increasingly popular in the United States. A recent Mayo Clinic study found that 61% of general medical patients reported the use of dietary supplements, most commonly multivitamins (41.5%), vitamin E (24%) and vitamin C (23%) (11). Among patients with cancer, 54% of those being treated at the University of Pennsylvania Cancer Center used unorthodox treatments, including diet therapy or megavitamins (12). This phenomenon is not limited to the United States; for example, a report from an Oncology Clinic in Norway described a 45% cumulative risk of being a user of nonproven therapy over a 5 year period (13). Unfortunately, at present little is known about the effects of combining chemotherapeutic agents and dietary manipulations (9).

The results described in this Annual Report explore the relationship between dietary variations in a specific nutrient, folic acid, and the toxicity of chemotherapeutic drugs. Most of the studies in this first year of the grant have focused on alkylating agents because these drugs are widely used in oncology patients and are particularly useful in patients with breast cancer. Our studies of the interaction of folate metabolism and alkylating agents at a molecular level have led to a greater understanding of these interactions and allowed us to propose a new model that can be tested. In addition, these studies indicate that correction of folic acid deficiency may protect against certain types of genetic damage during alkylator therapy. The types of damage observed in folate deficient cells treated with EMS, i.e., intragenic deletions and G>A transitions, are associated with the development of therapy-related acute leukemia in humans and of mammary carcinoma in rats (14,15). One could speculate that dietary supplementation

with folic acid during alkylator treatment might reduce the risk of developing these secondary malignancies.

Our studies at a cellular level suggest that folate metabolism can modulate GSH levels. This previously unrecognized relationship may help explain why dietary folate levels influence the efficacy and toxicity of alkylating agents (6). GSH levels are important determinants of alkylator activity by conjugating the nitrogen mustard group (3). The ability of tumor cells to lower the concentrations of aziridinium ions is a critical event in the development of resistance to alkylating drugs (3). Changes of intracellular GSH levels influence scavenging of the aziridinium ions formed. Modulation of GSH levels has been accomplished experimentally. GSH deficiency induced by buthionine sulfoximine sensitizes rodents to treatment with cyclophosphamide but causes severe mitochondrial damage (4). GSH levels have been stimulated by administration of GSH monoester to protect against cyclophosphamide toxicity, but the drug itself can cause toxicity (4). Changes in folic acid levels by dietary supplementation may represent a less toxic method to manipulate GSH levels and thereby modulate resistance to alkylating agents.

The *in vivo* studies in rats of the interaction of folate metabolism and chemotherapeutic drugs are at a relatively early stage. It appears that dietary folate levels affect the toxicity of cyclophosphamide, but that the relationship is more complex than originally thought. Our previous studies indicated that toxicity was inversely proportional to folate levels (6). The current studies suggest that animals maintained on either high or low folate diets have more toxicity than animals on a diet with a "standard" amount of folic acid. Therefore there may be an optimal amount of dietary folate to reduce the toxicity of alkylating agents. However, full interpretation of this data must await completion of the experiment and statistical analyses, as well as completion of the efficacy studies. It may be that manipulation of folate levels not only alters toxicity but enhances efficacy, leading to an improved therapeutic index.

Finally, the unexpected finding that rats on a Purified Diet were much more sensitive to the effects of cyclophosphamide than rats on a cereal-based diet emphasize that dietary changes can have a profound and largely unrecognized effect on chemotherapy. The mechanism(s) of this resistance to toxicity is unknown at present but may be related to quenching of reactive oxygen species or increased induction of the cytochromes P-450 system. It will be of interest to determine if cyclophosphamide has an altered efficacy in rats maintained on a cereal-based diet. Does this diet only reduce systemic toxicity, or does it also increase the resistance of the tumor to alkylating agents, thereby negating any improvement of the therapeutic index? During the coming year these studies will be performed as described in the Statement of Work.



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#### APPENDICES

## U.S. Army Medical Research and Materiel Command Animal Use Report

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Principal Investigator: \_\_\_\_\_

Principal Investigator: Richard F. Branda  
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Award Number: DAMD17-98-1-8345This Report is for Fiscal Year (01 October - 30 September): 98-99

COPY

AAALAC\* Accreditation Status (circle one): Full Provisional Not AccreditedDate of Last USDA Inspection: June 16, 1999 USDA Registration Number: 13-R-001

## Definitions of Column Headings on Back of Form

A. Animal	B. Number of animals purchased, bred, or housed but not yet used	C. Number of animals used involving no pain or distress	D. Number of animals used in which appropriate anesthetic, analgesic, or tranquilizing drugs were used to alleviate pain	E. Number of animals used in which pain or distress was not alleviated	F. Total Number of Animals (Columns C+D+E)
Dogs					
Cats					
Guinea Pigs					
Hamsters					
Rabbits					
Non-human Primates					
Sheep					
Pigs					
Goats					
Horses					
Mice					
Rats		4		202	206
Fish					
List Others:					

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# MUTATION RESEARCH

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Mutation Research 427 (1999) 79–87

The effect of folate deficiency on the *hprt* mutational spectrum in  
Chinese hamster ovary cells treated with monofunctional  
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Received 7 December 1998; received in revised form 16 April 1999; accepted 20 April 1999



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## The effect of folate deficiency on the *hprt* mutational spectrum in Chinese hamster ovary cells treated with monofunctional alkylating agents

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Received 7 December 1998; received in revised form 16 April 1999; accepted 20 April 1999

### Abstract

Folic acid deficiency acts synergistically with alkylating agents to increase DNA strand breaks and mutant frequency at the *hprt* locus in Chinese hamster ovary (CHO) cells. To elucidate the mechanism of this synergy, molecular analyses of *hprt* mutants were performed. Recently, our laboratory showed that folate deficiency increased the percentage of clones with intragenic deletions after exposure to ethyl methanesulfonate (EMS) but not *N*-nitroso-*N*-ethylurea (ENU) compared to clones recovered from folate replete medium. This report describes molecular analyses of the 37 *hprt* mutant clones obtained that did not contain deletions. Folate deficient cells treated with EMS had a high frequency of G > A transitions at non-CpG sites on the non-transcribed strand, particularly when these bases were flanked on both sides by G:C base pairs. Thirty-three percent of these mutations were in the run of six G's in exon 3. EMS-treated folate replete cells had a slightly (but not significantly) lower percentage of G > A transitions, and the same sequence specificity. Treatment of folate deficient CHO cells with ENU resulted in predominantly T > A transversions and C > T transitions relative to the non-transcribed strand. These findings suggest a model to explain the synergy between folate deficiency and alkylating agents: (1) folate deficiency causes extensive uracil incorporation into DNA; (2) greatly increased utilization of base excision repair to remove uracil and to correct alkylator damage leads to error-prone DNA repair. In the case of EMS, this results in more intragenic deletions and G:C to A:T mutations due to impaired ligation of single-strand breaks generated during base excision repair and a decreased capacity to remove *O*<sup>6</sup>-ethylguanine. In the case of ENU additional T > A transversions and C > T transitions are seen, perhaps due to mis-pairing of *O*<sup>2</sup>-ethylpyrimidines. Correction of folate deficiency may reduce the frequency of these types of genetic damage during alkylator therapy. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Folate; Alkylating agent; *hprt*; DNA repair

### 1. Introduction

The results of epidemiologic and interventional studies suggest that nutritional folic acid deficiency

promotes carcinogenesis. Interest has focused particularly on colon cancer, where retrospective case-control studies indicating that folate supplementation lowered the risk of colon cancer were confirmed by prospective studies in both men and women [1–4]. Experimental studies in rats found that nutritional folate deficiency enhanced the development, while increasing dietary folate content progressively re-

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duced the incidence of macroscopic colonic neoplasia after dimethylhydrazine treatment [5,6]. In addition, suggestive evidence has accumulated to implicate folate deficiency in the development of cancer at other sites in humans such as the uterine cervix, esophagus and brain (reviewed in Refs. [7,8]), and studies of dietary folate deficiency in mice described an increased incidence of leukemia after Friend Virus infection [9].

Although many mechanisms have been proposed to explain the promotion of carcinogenesis by dietary folate deficiency [7,8], most attention has concentrated on alterations of DNA synthesis and repair. Folic acid deficiency has several important effects on DNA metabolism. DNA synthesis is defective in megaloblastic anemia due to deficiency of either folic acid or vitamin B12, manifesting a reduced rate of replication fork movement [10,11]. Because the biosynthesis of DNA precursors is impaired in folate deficiency, there is inhibition of gap-filling and/or joining of Okazaki pieces, resulting in persistent single-stranded regions [10]. In addition, stalled replication forks are prone to double-strand breaks and induction of deletions [12,13]. Decreased synthesis of thymidylate due to a lack of the folate cofactor leads to extensive incorporation of uracil into DNA and strand breaks [14–16]. Double strand breaks and deletions may occur as a result of excision repair of opposing uracil residues by uracil–DNA glycosylase [17]. There is evidence that folate deficiency also impairs DNA repair. Thus, nucleotide pool imbalance, as is found in folate-deficient cells [18], inhibits the repair or enhances error-prone repair of spontaneous DNA damage [19,20], nutritional folate deficiency impairs the repair of  $\gamma$ -irradiation or ethyl methanesulfonate (EMS)-induced strand breaks [21], methotrexate inhibits DNA excision repair following exposure to ultraviolet irradiation or EMS [22], and folate depletion impairs DNA excision repair in rat colon [23]. Finally, diminished folate status alters the pattern of DNA methylation [24,25], rendering the hypomethylated regions more susceptible to nuclease attack and thereby resulting in DNA strand breaks [26,27].

Given the diverse and serious effects of folate deficiency on DNA metabolism, it is not surprising that numerous types of genetic damage have been reported in folate deficient cells. These include chro-

mosomal gaps and breaks, fragments, triradial, quadriradial and ring forms, allocyclic chromosomes, micronucleus formation, increased rates of sister chromatid exchanges, and expression of fragile sites (reviewed in Refs. [28,29]). In addition, our laboratory has shown that deficiency of this vitamin acts synergistically with alkylating agents to increase DNA strand breaks and mutant frequencies at the *hprt* locus both in vitro and in vivo [21,30]. To elucidate the mechanism underlying this synergy, we performed molecular analyses of *hprt* mutant CHO cells that were treated with EMS or *N*-nitroso-*N*-ethylurea (ENU). An analysis by multiplex PCR amplification of *hprt* exons from cells treated with EMS showed intragenic deletions in 9/46 (19.6%) clones derived from folate-deficient cells, but in none in 16 mutants isolated from cells grown in folate-replete medium [31]. In these experiments, the mutant frequency increased from  $618 \pm 61 \times 10^{-6}$  in folate replete medium to  $1796 \pm 165 \times 10^{-6}$  in low folate medium, a 200% increase. Since the mutant frequency for the folate replete cells was 1/3 of the mutant frequency in low folate, 1/3 of the mutations can be considered 'background' and 2/3 (67%) as low folate induced. Therefore, the 20% frequency of deletions in the mutants in the low folate cells only explains about 30% of EMS-induced mutations which are due to the low folate treatment. The number of deletions in mutants generated by ENU was equally low in folate-deficient and folate-replete clones (4% vs. 3.8%) and thus cannot explain the large mutant frequency differences between the folate replete and low folate cultures ( $838 \times 10^{-6}$  vs.  $307 \times 10^{-6}$ ) [31]. Because deletions cannot explain all of the increase in mutant frequency after EMS and especially after ENU treatment of low folate cells, we now describe the mutational spectrum at the *hprt* locus in mutants that did not display intragenic deletions. Our results indicate that the combination of folate deficiency and EMS produces *hprt* mutants with a high frequency of G > A transitions at non-CpG sites on the non-transcribed strand, particularly when these bases are flanked on both sides by G:C base pairs. Folate deficient cells treated with ENU were found to have high proportions of C > T transitions and T > A transversions, but surprisingly few G > A transitions on the non-transcribed strand.

## 2. Materials and methods

### 2.1. Cell culture

Clones of the CHO-K1 cell line were obtained as described previously [21,31] and cryopreserved in liquid nitrogen. Experiments were carefully scrutinized so that only clones from independent cultures were included in the final analysis. Clones were quick thawed in a 37°C water bath and added to Ham's F-12 medium (JRH Biosciences, Lenexa, KS) containing 10% calf bovine serum (Hyclone Laboratories, Logan, UT) in a 75 cm tissue culture flask and placed in a 37°C, 5% CO<sub>2</sub> incubator. Cells were

allowed to attach overnight, then covered with fresh medium, and grown for 3–4 days until confluent. The cells were then trypsinized, counted and aliquoted to eight 0.5 ml tubes, six at  $1 \times 10^4$  and two at  $5 \times 10^4$  cells, and stored at –70°C. The remaining cells were frozen in liquid nitrogen 1.2 ml cryotubes in 1 ml of 8% DMSO, 50% serum, 42% Ham's F-12 medium.

### 2.2. cDNA Synthesis, PCR amplification and DNA sequencing

A master mix was made for cDNA synthesis prior to obtaining dry cell pellets from the –70°C freezer.

Table 1  
Oligonucleotide primers used for PCR analysis of CHO DNA

RT-PCR primers		
Name	Sequence	Base numbers <sup>a</sup>
215 +	ACCTCACCGCTTTCTCGTGC	Ex1 – 88 to Ex1 – 68
216 –	AAGCAGATGGCTGCAGAACT	IVS9 + 122 to IVS + 141
ZEE-1 +	GGCTTCCTCCTCACACGCT	Ex1 – 50 to Ex1 – 31
VRL16 –	GCAGATTCAACTTGAATTCTCATC	IVS9 + 58 to IVS9 + 82
Multiplex primers		
Name	Sequence	Base numbers <sup>a</sup>
Exon 1 +	TCTGCAGGCTTCCTCCTCACACCG	Ex1 – 56 to Ex1 – 33
Exon 1 –	ACATGTCAAGGCAACGCCATTTCCA	IVS1 + 301 to IVS1 + 326
Exon 2 +	AGCTTATGCTCTGATTGAAATCAGCTG	IVS1 – 42 to IVS1 – 15
Exon 2 –	ATTAAGATCTTACTTACctgtccaraatc	Base 123 (exon 2) to IVS2 + 29
Exon 3 +	CCGTGATTTTATTTTGTAGgactgaaag	IVS2 – 28 to base 143 (exon 3)
Exon 3 –	TACATACAAAACCTAGGATTGCCATATT	IVS3 + 30 to IVS3 + 57
Exon 4 +	TGTGTGTATTCAAGAATATGCATG	IVS3 – 48 to IVS3 – 24
Exon 4 –	CCAAGTGAGTGATTGAAAGCACAG	IVS4 + 57 to IVS4 + 81
Exon 5 +	AACATATGGGTCAAATATTCTTTCTAATAG	IVS4 – 161 to IVS4 – 132
Exon 5 –	GCTGAGAAAAATTTAACAGTATTTTAG	IVS5 + 19 to IVS5 + 46
Exon 6 +	ACTTACCACCTACCATTAAATACC	IVS5 – 41 to IVS5 – 17
Exon 6 –	AAGCAATTGCTTATTGCTCCCAATG	IVS6 + 36 to IVS6 + 61
Exon 7,8 +	GTTCTATTGTCTTTCCCATATGTC	IVS6 – 49 to IVS6 – 25
Exon 7,8 –	TCAGTCTGGTCAAATGACGAGGTGC	IVS8 + 88 to IVS8 + 113
Exon 9 +	CAATTCTCTAATGTTGCTTACCTCTC	IVS8 – 69 to IVS8 – 42
Exon 9 –	GACAATCTATCGAAGGCTCATAGTGC	Ex9 + 180 to Ex9 + 206
Long PCR primers		
Name	Sequence	Base numbers
S2 +	TGCTCTGATTGAAATCAGC	IVS1 – 33 to IVS1 – 17
S3 –	CATACAAAACCTAGGATTGCC	IVS3 + 34 to IVS3 + 53

<sup>a</sup>The bases listed as Ex1 – X to – Y are 5' (upstream) to the 'A' of the ATG start codon, i.e., Ex1 – 88 to – 68 is the sequence from 88 bases 5' to the 'A' of the ATG start codon to 68 bases 5' of that 'A'. Base 123 and base 143 in the exon 2 antisense and exon 3 sense primers, respectively, are cDNA base numbers. The base listed as IVSX – Y is Y bases 5' to the 3' end of intron X while the base listed as IVSX + Y is Y bases 3' of the start of intron X. The bases listed as Ex9 + N are N bases 3' to the A of the TGA stop codon.



Cells were kept on dry ice until the addition of a mix containing 0.5  $\mu$ l NP-40 (Sigma, St. Louis, MO), 4  $\mu$ l 25 mM  $MgCl_2$ , 2  $\mu$ l PCR II Buffer (Perkin-Elmer/Cetus, Norwalk, CT), 8  $\mu$ l 2.5 mM dNTP's (Perkin-Elmer), 1  $\mu$ l Reverse Transcriptase (Murine Moloney Leukemia Virus, Perkin Elmer), 1  $\mu$ l RNase inhibitor (Perkin-Elmer), 1  $\mu$ l Oligo (dT) (Perkin-Elmer) and 2.5  $\mu$ l HPLC water (Sigma). Cells were thawed in the reaction mix and cDNA was generated in a Perkin-Elmer 2400 Thermal Cycler with a profile of 42°C for 15 min and 99°C for 5 min. Amplification of the cDNA was done in two rounds of nested PCR. A 5  $\mu$ l aliquot of cDNA was transferred into a first round mix of 1.9  $\mu$ l 25 mM  $MgCl_2$ , 2.45  $\mu$ l PCR II Buffer (Perkin-Elmer), 2  $\mu$ l 2.5 mM dNTP's (Perkin-Elmer), 17.25  $\mu$ l HPLC water (Sigma), 0.125  $\mu$ l each 30 pmole forward (215 +) and reverse (216 -) primers (Gibco BRL Life Technologies, Grand Island, NY) and 0.15  $\mu$ l Amplitaq polymerase (Perkin-Elmer). The second round amplification consisted of 3  $\mu$ l 25 mM  $MgCl_2$ , 5  $\mu$ l PCR II Buffer (Perkin-Elmer), 4  $\mu$ l 2.5 mM dNTP's (Perkin-Elmer), 36.25  $\mu$ l HPLC water (Sigma), 0.25  $\mu$ l each 30 pmole forward (ZEE1 +) and reverse primers (VRL16 -) (Gibco BRL, Life Technologies), 0.25  $\mu$ l Amplitaq polymerase (Perkin-Elmer) and 1  $\mu$ l of the first round product. A PCR profile of 94°C–1', 60°C–1', 72°C–1'30" was used for both rounds. The final product was run on a 1.5% Agarose (Gibco BRL, Life Technologies) gel, stained with ethidium bromide and observed under UV light. If multiple products were obtained (an exon 2 + 3 deletion product can often be seen even in wildtype cells), the largest product was excised, Gene Cleaned (Bio 101, Vista, CA) and sequenced with an ABI 373 sequencer.

Genomic PCR was performed as described previously for multiplex PCR [31] except using only the primer pair for the exon of interest (primers are listed in Table 1).

The Long PCR method consisted of combining 45  $\mu$ l HPLC water, 3  $\mu$ l 10 mM dNTPs, 1  $\mu$ l 50 pM forward (S2 +) and 1  $\mu$ l 50 pM reverse (S3 -) primer and a single wax bead (Ampliwax, Perkin-Elmer) (Table 1). The mixture was heated to 80°C–5' then 25°C–5' followed by addition of 10  $\mu$ l Long PCR Buffer 2 (Perkin-Elmer), 0.75  $\mu$ l Enzyme (Platinum Taq, Perkin-Elmer), 1  $\mu$ l DNA template

and 38.25  $\mu$ l HPLC water. An initial cycle of 95°C–1'30", 58°C–30", 68°C–8' followed by 40 cycles of 94°C–15", 58°C–30", 68°C–8' with a 10' extension at 68°C was used on the Perkin-Elmer 2400 Thermal Cycler.

### 3. Results and discussion

DNA sequence analysis was performed on 13 *hprt* mutant clones collected after EMS treatment of folate deficient CHO cells, nine clones produced by EMS treatment of folate replete cells, nine clones from ENU-treated folate deficient cells, and six clones obtained after ENU treatment of folate replete cells. None of these clones had previously demonstrated intragenic deletions after multiplex PCR amplification of *hprt* exons. Initially, RT-PCR and cDNA sequencing was performed. Fourteen mutants lacked a whole exon(s) by RT-PCR. Two EMS folate replete mutants were missing exon 2 and two were missing exons 2 + 3. One EMS folate deficient mutant was missing exons 2 + 3 and one was missing exons 2 to 6. Three ENU folate replete mutants were missing exons 2 + 3, one was missing exon 3 and one was missing exon 5. One ENU folate deficient mutant was missing exon 8 and two were missing exons 2 + 3. We then used genomic PCR of the relevant exon(s) utilizing the CHO multiplex primers (Table 1, Ref. [32]) followed by automated sequencing to attempt to define the mutation causing the splice alteration in these mutants. This was a successful approach for three mutants. However, for the exon 2 or 3 loss mutants, while we could sequence the splice acceptor sequence for exon 2 and the splice donor sequence for exon 3, the primers overlapped the other splice site. Because there is limited intronic sequence information available for the CHO *hprt* gene, we were unable to synthesize the more appropriate primers. We did utilize long PCR using new exon 2 sense (S2 +) and exon 3 antisense (S3 -) primers. However, we were still only able to successfully PCR three of the mutants and of these three only one mutation (in the exon 3 acceptor) was determined despite repeated attempts.

The results of the sequencing studies are shown in Table 2. With the exception of a single splice site mutation in exon 6, all of the characterized mutations were in either exon 3 or exon 8. Exon 3 is the largest

Table 2

Base pair changes in the *hprt* gene of 6-thioguanine resistant CHO cells exposed to EMS or ENU when the cells were folate replete or deficient

EMS + folate			EMS – folate		
Mutation	Target sequence	Codon and amino acid change	Mutation	Target sequence	Codon and amino acid change
209G > A (exon 3)	AAG GGG GGC	70Gly > Glu	197G > A (exon 3)	CTC TGT GTG	66Cys > Tyr
389T > A (exon 5)	AAT GTC TTG	130Val > Asp	200T > G (exon 3)	TGT GTG CTG	67Val > Gly
551C > T (exon 8)	ATT CCA GAC	184Pro > Leu	208G > A (exon 3)	AAG GGG GGC	70Gly > Arg
574G > A (exon 8)	TAT GCC CTT	192Ala > Thr	208G > A (exon 3)	AAG GGG GGC	70Gly > Arg
574G > A (exon 8)	TAT GCC CTT	192Ala > Thr	208G > A (exon 3)	AAG GGG GGC	70Gly > Arg
			209G > A (exon 3)	AAG GGG GGC	70Gly > Glu
			485G > A (exon 6)	CCA GAC TTT	177Asp > Glu (splice 6)
			551C > T (exon 8)	ATT CCA GAC	184Pro > Leu
			568G > A (exon 8)	GTT GGA TAT	190Gly > Arg
			569G > A (exon 8)	GTT GGA TAT	190Gly > Glu
			[exclusion of exons 2–6]		
			575C > A (exon 8)	TAT GCC CTT	192Ala > Asp
			575C > A (exon 8)	TAT GCC CTT	192Ala > Asp
ENU + folate			ENU – folate		
Mutation	Target sequence	Codon and amino acid change	Mutation	Target sequence	Codon and amino acid change
74C > T (exon 2)	ATT CCT CAT	38Pro > Leu	389T > A (exon 5)	AAT GTC TTG	130Val > Asp
IVS4-IG > A	ttctagAAT	Intron (splice exon 5)	389T > A (exon 5)	AAT GTC TTG	130Val > Asp
			416C > T (exon 6)	GAC ACT GGT	139Thr > Ile
			464C > T (exon 6)	AAG CCC CTC	155Pro > Leu
			464C > T (exon 6)	AAG CCC CTC	155Pro > Leu
			Δ534–536 (exon 8)	TTT [GTT] GGA	Δ165Val
			IVS3-9T > G <sup>a</sup>	ttt att ttt	Intron (splice exon 2,3) (new ag splice site)

<sup>a</sup>Splice junction sequenced using the Long PCR method (see Section 2).

exon and exons 3 and 8 the most frequently mutated exons in the human *HPRT* gene [33]. Particularly susceptible to mutation were guanine bases in exon 3 where six guanines are found in a row (cDNA bases 207–212).

EMS follows a mixed  $S_N1/S_N2$  type reaction and is believed to be mutagenic by reaction with the  $O^6$  position, and, to a lesser extent, to the  $N^7$  position of guanine [34,35]. The  $N$ -alkylation products yield apurinic sites that are processed further by base excision repair and may cause mutations by mis-incorporation or error-prone repair processes [35–38]. The  $O^6$ -ethylguanine is mutagenic through direct miscoding by pairing with thymine during

replication [35,39]. The spectrum of EMS is dominated by G > A transitions, and EMS has a relatively low efficiency for deletions in exponentially growing cells [35].

In the EMS treated cells, both the folate replete and folate deficient grown cells showed a high percentage of GC:AT mutations (80% and 75%, respectively). The major mutagenic lesion for EMS is  $O^6$ -ethylguanine which is directly mutagenic and is not removed in CHO cells because they do not contain  $O^6$ -alkylguanine–DNA alkyltransferase (AGT) [40,41]. Assuming that the G is the mutagenic target, then three of the four (75%) GC:AT mutations in the folate replete cells and eight of the nine

(89%) GC:AT mutations in the low folate cells occurred on the non-transcribed strand. Op het Veld et al. [42] also studied EMS treated CHO9 cells (folate replete) and found that 78% (14/18) of point mutations were at GC:AT basepairs with 64% on the non-transcribed strand (seven mutations were not determined in genomic DNA). When they studied a DNA repair defective cell line, EM-C11, they obtained 21/22 (95%) GC:AT mutations among the point mutations with 16/21 (76%) on the non-transcribed strand (nine mutants were deletions, two were frameshifts and seven mutants were undefined). The large proportion of deletions were ascribed to the DNA repair defect in the EM-C11 cell line similar to our observations of a larger percentage of deletions in EMS-treated folate deficient cells [39]. Our result of 89% G > A transitions in the folate deficient cells therefore suggests that low folate may also increase the proportion of mutations occurring on the non-transcribed strand.

In the studies here, of the eight G > A transitions on the non-transcribed strand in the low folate cells, six were flanked 3' by a purine and four were flanked on both sides by G:C base pairs. One of the three G > A transitions in the EMS folate replete cells was flanked by a 3' purine (G) (at 209 in the run of six G's). Op het Veld et al. [42] also found a high proportion of 3' purines (8/9-CHO9 and 13/16-EM-C11) in their experiments.

The sequence specificity of G > A transitions may reflect the fact that excision-repair enzymes are less efficient at removing *O*<sup>6</sup>-ethylguanine if this adduct is flanked by G:C base pairs [34]. However, excision repair is known to be less effective in rodent than human cells to remove *O*<sup>6</sup>-ethylguanine lesions and therefore may have relatively little to do with the sequence specificity in CHO cells [42,43]. On the other hand, nucleotide excision repair may be active in folate deficient cells, since nucleotide excision repair is involved in removal of *N*-ethylpurines from the transcribed strand, and folate deficient cells showed the expected bias to mutations in the non-transcribed strand [43–45]. This strand bias does not appear to be due to a highly disproportionate number of mutable bases on the non-transcribed strand, since there were 120 mutable guanines (that is, guanines that if mutated would result in an amino acid change) on the non-transcribed strand (59%) and 83 mutable

guanines on the transcribed strand (41%). Studies in cells that have *O*<sup>6</sup>-alkylguanine–DNA alkyltransferase have lower levels of EMS induced mutations and do not show a strand bias in GC:AT mutations [46].

The molecular analyses of EMS-treated folate deficient CHO cells resemble the findings in the base excision repair defective cell line EM-C11 in that both have G > A transitions as the largest class of *hprt* mutations and both have high fractions of deletion mutations [44,47]. These similarities suggest that folate deficiency may impair base excision repair and are consistent with reports that nutritional folate depletion impairs DNA excision repair in rat colon [23], and that methotrexate inhibits DNA excision repair after exposure to EMS or ultraviolet irradiation [22].

A possible mechanism for impaired DNA excision repair in folate-deficient cells is mispairing and overloading of the repair system by excessive incorporation of uracil into DNA. Folate deficiency limits the methylation of dUMP to dTMP, resulting in extensive incorporation of uracil into DNA by DNA polymerase [14–16]. Repair of uracil residues utilizes excision repair [17,48]. Thus, a model to explain the synergistic interaction of EMS and nutritional folate deficiency, and the resulting mutational spectra, can be proposed: folate deficiency increases uracil incorporation into DNA, thereby increasing utilization of base excision repair and in turn promoting defective DNA repair in EMS-treated cells. This results in a higher number of intragenic deletions and G > A mutations due to impaired ligation of single-strand breaks generated during base excision repair and a reduced capacity to remove *O*<sup>6</sup>-ethylguanine.

ENU reacts by an S<sub>N</sub>1 mechanism and produces significant levels of alkylation of oxygens, such as the *O*<sup>6</sup>-position of guanine and the *O*<sup>2</sup> and *O*<sup>4</sup> positions of thymine, resulting in miscoding or misrepair [34,35]. The mutational spectrum in ENU-treated mammalian cells is predominantly G > A and to a lesser extent A > G transitions and T > A transversions [35,49,50]. The latter type of mutation may reflect thymidine incorporation opposite *O*<sup>2</sup>-ethylthymine [50].

The interpretation of the molecular analyses in ENU-treated CHO cells is limited by the large proportion of clones that had splice site mutations that

could not be fully characterized (the actual base change could only be determined in two mutants, which made statistical comparisons between the folate replete and deficient groups impossible). These mutations must be in the exon 2 donor or exon 3 acceptor sites that could not be sequenced for technical reasons. It is unclear why five of the six (83%) ENU folate replete mutations would be in splice sites unless a hotspot(s) exists in these regions. Four of the nine (44%) EMS folate replete mutations are also splice site mutations; however, splice site mutations make up only 2/13 (15%) and 3/9 (33%) of the EMS folate deficient and ENU folate deficient mutations, respectively. We previously reported that intragenic deletions were relatively uncommon in both folate deficient and replete cells after ENU exposure [31]. The spectrum of the remaining mutants was mainly C > T transitions and T > A transversions relative to the non-transcribed strand (Table 2).

In cells with *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase, ENU-induced mutations are predominantly TA:AT (38–45%), with a very strong bias for T > A transversions on the non-transcribed strand [46,50–53]. T > C and T > G mutations each make up ~ 15% of the mutations and G > A and C > T mutations each about 7%. In CHO cells, Op het Veld et al. [42] found 22% T > A (13/59), 8% T > C (5/59), 10% T > G (5/59) and 32% G > A (19/59) and 10% C > T (6/59). Our spectrum in folate deficiency, though very small, found 33% (2/6) T > A, 0% T > C, 17% (1/6) T > G, 0% G > A and 50% (3/6) C > T. This is a very high percentage of C > T which is not seen in AGT competent or incompetent cells. The ratio of G > A:C > T is also much different with a 3:1 ratio seen in the previous study of folate replete CHO cells and an approximately 1:1 study in AGT competent cells.

Consistent with prior observations by others, T > A transversions in our clones occurred preferentially at a thymine with an adjacent purine at its 5' site and on the non-transcribed strand [50]. C > T transitions are distinctly unusual in ENU-treated mammalian cells [35,49,50]. One mechanism proposed for C > T transitions is deamination of cytosine to uracil under conditions of limited availability of *S*-adenosylmethionine [54], as occurs in folate deficiency. Uracil then codes for thymine during DNA replication [54]. In addition, decreased levels of *S*-adenosylmethionine enhance binding of DNA methyltransferase, thereby inhibiting repair of uracil [54,55].

Although an attractive hypothesis, this mechanism may not apply to our experimental results since the cytosines involved were not at CpG sites, and hence may not have been methylated. Alternatively, *O*<sup>2</sup>-ethylcytidine may act as uracil and code for thymine [56] giving the pathway *O*<sup>2</sup>-ethylcytidine → U → T. This mechanism also would promote saturation of base excision repair by utilizing uracil-DNA glycosylase.

The studies reported here will require confirmation in a larger number of clones. Study of approximately 35–50 mutants per group would be required to detect a 30–40% difference in frequency of one mutation type at 80% power, while it would require over 500 mutants per group to reach significance for the 7% difference in frequency in G > A mutations seen here between the EMS + folate and EMS – folate groups. Approximately 120 mutations per group would be needed to verify the high proportion (89%) of mutations occurring on the non-transcribed strand. These preliminary results suggest, however, that correction of folate deficiency may protect against certain types of genetic damage during alkylator therapy. In addition, similar studies in other cell types would be of interest. The mutational spectrum after alkylator treatment in CHO cells probably is not representative of many other mammalian cell types because CHO cells lack alkyl transferase [40,41]. Therefore, studies of the effect of folate deficiency on the mutational spectrum in lymphocytes from rats treated with alkylating agents [30] and in human lymphocytes are planned. Studies in human cells may be useful both to provide additional insights into the synergy between folate deficiency and mutation induction and for molecular epidemiologic approaches to elucidate the relationship between dietary components such as folic acid and environmental mutagens or carcinogens.

#### Acknowledgements

Supported by grants from the National Cancer Institute, Bethesda, MD (CA 41843) and the Department of the Army (DAMD17-98-1-8345). The content of the information does not necessarily reflect

the position or policy of the U.S. government, and no official endorsement should be inferred. Automated sequencing was performed in the Vermont Cancer Center DNA Analysis Facility which is supported by NCI P30 CA 22435.

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**Publication information:** *Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis* (ISSN 0027-5107). For 1999 volumes 421-431 are scheduled for publication. Volumes 421 and 422 were published ahead of schedule in 1998 but remain part of the 1999 subscription year. Subscription prices are available upon request from the Publisher. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by surface mail except to the following countries where Air delivery via SAL mail is ensured: Argentina, Australia, Brazil, Canada, Hong Kong, India, Israel, Japan, Malaysia, Mexico, New Zealand, Pakistan, PR China, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. For all other countries airmail rates are available upon request. Claims for missing issues should be made within six months of our publication (mailing) date.

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# Nutritional Folate Status Influences the Efficacy and Toxicity of Chemotherapy in Rats

By Richard F. Branda, Elizabeth Nigels, Amy R. Lafayette, and Miles Hacker

The effect of folate status on the efficacy and toxicity of chemotherapy was investigated in weanling Fischer 344 rats maintained on diets of varying folate content or supplemented with daily injections of folic acid, 50 mg/kg, for 6 to 7 weeks. MADB106 rat mammary tumor growth rate was the same in folate replete and supplemented rats, but retarded in the low folate groups. The tumor growth inhibitions in low folate, replete and high folate rats treated with cyclophosphamide were: 53%, 98%, and 97% ( $P = .048$ ); with 5-fluorouracil (5-FU): 46%, 49%, and 66%; and with doxorubicin: 25%, 55%, and 61%. Significant differences in survival were observed

for cyclophosphamide ( $P = .0084$ ) and 5-FU ( $P = .025$ ) related to dietary folate content. Thus, folate deficiency impedes tumor growth rate, but supplementation does not accelerate it in folate replete animals. Correction of folate deficiency approximately doubles the efficacy of cyclophosphamide in rats with much less host toxicity. Folate repletion improves survival in 5-FU-treated animals. These studies indicate that nutritional folate status has an important influence on the efficacy and toxicity of some commonly used cancer chemotherapeutic drugs.

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**F**OLIC ACID DEFICIENCY has been observed in patients with cancer due to the combination of inadequate dietary folacin content, poor dietary intake, and the increased metabolic needs imposed by malignancy.<sup>1</sup> For example, Magnus<sup>2</sup> reported that in a series of patients with metastatic cancer, 85% had abnormally low serum folate levels and 16% had low red blood cell folate levels. Clamon et al,<sup>3</sup> in a study of lung cancer patients, noted that 36.7% of patients with limited stage disease, 38.5% of patients with hepatic metastases, and 47.1% of patients who had lost more than 9% of body weight had reduced serum folate levels. However, clinicians are uncertain about the management of folic acid deficiency in patients with cancer. In 1948, Farber et al<sup>4</sup> described acceleration of the leukemic processes by folic acid conjugates to a degree not encountered in other children with acute leukemia, and Heinle and Welch<sup>5</sup> reported that administration of folic acid to three patients with chronic myeloid leukemia was attended by rapid hematologic and clinical relapse in each case. Subsequent studies in rodents confirmed that dietary restriction of folic acid inhibited the growth of tumors. Rosen and Nichol<sup>6</sup> reported that when rats were placed on a folate-deficient diet 2 weeks before transplantation of Walker carcinosarcoma 256, tumor growth was inhibited by 95% on the 28th day after transplantation. The tumor remained viable and resumed rapid growth when folate was added to the diet.<sup>6</sup> Potter and Briggs<sup>7</sup> similarly found that dietary folate deficiency in mice retarded the growth of ascites tumor cells of lymphocytic neoplasms. An attempt to extend these animal studies to humans by placing seven patients with advanced malignancies on a folate-deficient diet for periods ranging from 25 to 140 days was unsuccessful, as no antitumor effect was noted.<sup>8</sup> Nevertheless, clinicians generally have been reluctant to administer folic acid to deficient cancer patients on the chance that the vitamin might accelerate the growth of the malignancy.

Recently, folic acid deficiency during early pregnancy has been associated with birth defects. Consequently the Food and Drug Administration has proposed that the diet of all Americans be fortified with 140 µg of folic acid per 100 g of cereal-grain product.<sup>9</sup> The effect of this dietary supplementation with folic acid on cancer patients is unclear, but of concern. The influence of subnormal or supranormal levels of folic acid on the efficacy and toxicity of chemotherapy has received relatively little attention, despite the fact that substantial numbers of patients with cancer are likely to have a nutritional abnormality of folate

metabolism or are taking supplemental vitamins, often in megadoses.<sup>10</sup> Therefore, the studies described herein were designed to investigate the interaction of nutritional folate status and response to chemotherapy.

## MATERIALS AND METHODS

**Animals.** Weanling female Fischer 344 rats (weighing approximately 60 g) were obtained from Charles River Canada (St-Constant, Quebec). The rats were maintained in groups of three or four for 10 days and fed standard rat chow (Teklad 7012; Harlan Teklad, Madison, WI). Then rats were housed individually in stainless steel wire-bottomed cages. Their diets were either folate replete (AIN-93G Purified Rodent Diet with Vitamin Free Casein containing 2 mg folic acid/kg of diet), low folate (AIN-93G with vitamin mix lacking folic acid), or very low folate (AIN-93G with vitamin mix lacking folic acid and with 1% succinyl sulfathiazole), all obtained from Dyets, Inc (Bethlehem, PA). At the completion of the study, the rats were anesthetized with pentobarbital sodium (60 mg/kg intraperitoneally [IP]) and exsanguinated by cardiac puncture. Samples were collected for subsequent folate analyses using previously described methods.<sup>11,12</sup> Approximately two thirds of blood removed was allowed to clot at room temperature, the serum was then separated by centrifugation and frozen in 100 µL aliquots at  $-70^{\circ}\text{C}$ . The remaining one third was added into a tube containing EDTA to prevent clotting, diluted 1:9 with distilled water containing 1% ascorbic acid, and frozen at  $-70^{\circ}\text{C}$  in 1-mL aliquots. The liver was weighed and 1 g homogenized in 3 vol 140 mmol KCl/L. The homogenate was then diluted 1:9 with 50 mmol potassium phosphate/L, pH 4.8, containing 1% ascorbic acid, and incubated for 24 hours at  $37^{\circ}\text{C}$  to allow endogenous conjugase to convert folate polyglutamates to monoglutamates. Afterwards, the homogenates were autoclaved, cooled on ice, and centrifuged at 2,000g for 10 minutes. The supernatants were frozen in three 1-mL aliquots at  $-70^{\circ}\text{C}$ . All animal

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Submitted March 10, 1998; accepted June 2, 1998.

Supported by Grants No. CA 41843 and P30CA22435 from the National Cancer Institute, Bethesda, MD.

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0006-4971/98/9207-0002\$3.00/0



protocols and care were approved by the Institutional Animal Care and Use Committee of the University of Vermont.

**Folate assay.** The tissue folate levels were measured on the aforementioned frozen samples by an assay that uses a bacteria that grows only in the presence of folate.<sup>13,14</sup> The growth turbidity of *Lactobacillus casei* (*L. casei*), which grows in proportion to the amount of folic acid present, was measured in a 96-well plate on a microplate reader.<sup>13</sup> A standard curve ranging from 0.0625 to 2 ng/well was made using folinic acid, as it is more stable than folic acid.<sup>13</sup> Tissue samples were diluted as needed to fit within the parameters of the standard curve. The standard curve, as well as the samples, were diluted in Sorenson's phosphate buffer with 1 mg/mL of ascorbic acid, pH 6.3.<sup>13</sup> Double strength maintenance media (DIFCO, Detroit, MI) with .5 mg/mL ascorbic acid was added to each plate at 150  $\mu$ L/well. The ascorbic acid was added to double strength maintenance medium and Sorenson's buffer and filter sterilized just before plating. *L. casei* was diluted 7:1 with Sorenson's phosphate buffer and added at a concentration of 80  $\mu$ L/well. The addition of 80  $\mu$ L/well of either sample, folate for the standard curve, or blank diluent brought the total volume to 310  $\mu$ L/well. The plates were read at a wavelength of 595 nm at 24 hours and sample concentrations were determined by linear regression.

**Tumor cell line.** The MADB106 rat mammary tumor cell line (obtained from Dr John Holcenburg, Department of Surgery, UCLA, Los Angeles, CA) was initially developed by IP injections of 7,12-dimethylbenz[ $\alpha$ ]anthracene into Fischer rats. Tumor was excised and minced. Tumor cells were separated from tumor stroma and injected into the right hind flank of the rats. Tumors were measured at two perpendicular planes using a tissue caliper. Tumor volumes were calculated from the formula:  $TV = 4/3 \pi r^3$  where  $r = (\text{diameter } 1 + \text{diameter } 2)/4$ . Tumor growth inhibition (TGI) was calculated:  $TGI = 1 - TV_{\text{treatment}}/TV_{\text{control}} \times 100$ .

**Statistical methods.** Analysis of variance was used to test the significance of differences in hematocrit levels, rat weights, blood, tumor and liver folate levels, and the efficacy of chemotherapy as influenced by folate status. If a significant F value was found, Fisher's least significant difference test was used to compare means. Tumor size and day were transformed with a natural log function. Hierarchical linear modeling procedures were used to examine the data.<sup>15</sup> At the first stage, each rat gave rise to a linear regression line between Log (Tumor Size) and Log (Day) with an intercept and slope. At the second stage, these intercepts and slopes were examined as a function of the four diet groups. The slopes of the linear regression lines were compared using a Fisher protected t type approach.<sup>15</sup> Rat survival in the toxicity study was analyzed by the Log-Rank test.

## RESULTS

Our intent in these experiments was to study a range of folate status in mammals, but to avoid extreme folate deficiency and thereby more closely approximate the typical clinical situation. Rats maintained for 6 weeks on the folate replete diet, the low folate diet, the very low folate diet, or the folate replete diet supplemented with folic acid, 50 mg/kg dissolved in 8.4% sodium bicarbonate solution and injected IP daily, grew at the same rate (the mean weights of the groups were within 2 g of each other on day 42) and were not anemic, but developed evidence of progressively severe tissue deficiency of the vitamin (Fig 1). There was a significant difference among the three dietary groups ( $P \leq .005$ ) for all three tissues, except between low folate and very low folate serum samples ( $P = .07$ ).

After 6 weeks on the special diets described above, the four groups of six animals each were inoculated subcutaneously with MADB106 rat mammary tumor ( $1 \times 10^5$  viable cells in a



Fig 1. Folate levels, as determined by the *L. casei* method, in whole blood, serum, and liver in rats maintained for 6 weeks on a folate replete diet containing 2 mg/kg folic acid (□), a low folate diet containing no folate (▨), or a very low folate diet consisting of no folate plus 1% succinyl sulfathiazole (▤). There was a significant difference ( $P \leq .005$ ) among the three dietary groups for all three tissues, except between low folate and very low folate serum samples ( $P = .07$ ). SD, standard deviation.

0.2-mL suspension). The rats were examined daily and, when palpable, the tumors were measured in 2 dimensions. Figure 2 shows that tumor growth rate slowed progressively with decreasing amounts of folate intake. Comparison of the slopes of the linear regression line between Log (Tumor Volume) and Log (Day) showed that the rate of tumor growth over time is diet-dependent ( $P < .05$ ), and that folate supplementation, even at the high levels used in these experiments, did not significantly increase tumor growth rate compared with tumors in folate replete rats (inset, Fig 2). However, folate deficiency of moderate or marked degree significantly retarded the tumor growth rate compared with folate replete or supplemented animals.

The folate levels in the tumors and livers from animals in the different folate groups are shown in Table 1. Both tumor and liver folate levels progressively decreased with decreasing dietary folate intake, but liver folate was higher than tumor folate in each dietary group. All tumor folate levels were significantly different from each other, at  $P \leq .05$ , except for the low folate versus very low folate groups. Similarly the differences between hepatic folate levels were significant ( $P \leq .02$ ) except for the low folate versus very low folate groups. The tumor and liver folate levels were correlated, with  $r^2 = 0.93$ ,  $P < .05$ .

Histologic examination of the tumors from the different folate groups showed poorly differentiated carcinoma, with abundant necrosis, and variable numbers of mitotic figures. There was no evidence of megaloblastosis. No pathologic distinction could be discerned among tumors differing in folate status.

To investigate the effect of folate status on the efficacy of chemotherapy, weanling Fischer 344 rats were divided into three groups of six animals each and maintained on a low folate

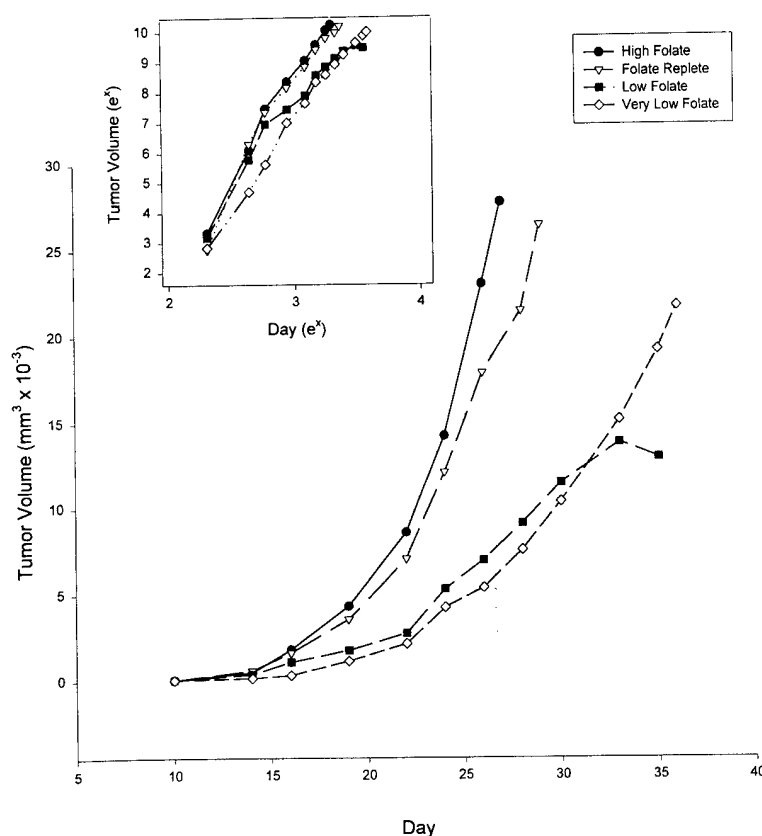


Fig 2. Growth of MADB106 mammary tumor in rats maintained on diets of varying folate content. After 6 weeks on the dietary conditions indicated, tumor cells were injected subcutaneously into six animals per group. Tumor volumes were measured by calipers. Insert: Tumor volume and day plotted on natural log scales. At a significance level of 5%, the slopes for the high folate diet were larger than the low and very low folate diets, but not the folate replete diet. The slopes for the folate replete diet were larger than the low folate diet, but not the very low folate diet. The slopes for the very low folate diet were larger than the low folate diet as a group.

diet, a folate replete diet, or the latter diet supplemented with folic acid, 50 mg/kg IP daily. After 7 weeks, MADB106 Mammary Tumor was injected subcutaneously ( $5 \times 10^5$  cells/0.5 mL). When tumor was palpable, the rats received cyclophosphamide 50 mg/kg, doxorubicin 5 mg/kg, 5-fluorouracil (5-FU) 50 mg/kg, or 0.9% NaCl solution IP. The planned course of treatment was to repeat the same medications 4 and 8 days later. However, we found that the folate-deficient rats were much more sensitive to the side effects of chemotherapy. After only two of the planned three treatments, the condition of the animals in this group rapidly deteriorated and the third treatment was not given. Of the folate-deficient animals treated with two doses of cyclophosphamide, two of six died (33% mortality). In contrast, there was a 33% mortality after three courses of cyclophosphamide in the folate-replete group, but no mortality in the folate-supplemented rats treated with three injections of cyclophosphamide. After three injections of 5-FU, there was one death in the folate-supplemented group (17% mortality), but none in the folate-deficient or replete dietary groups. There

were no deaths among the three dietary groups treated with doxorubicin.

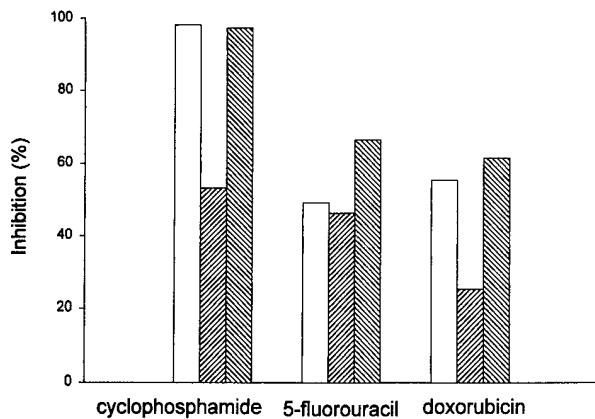
To compare the efficacy of chemotherapy under different dietary conditions, tumor inhibition as a percentage of control (0.9% NaCl solution-injected rats) was calculated 96 hours after the second injection of chemotherapy (just before the scheduled third injection). Figure 3 shows that the TGIs for cyclophosphamide were 53%, 98%, and 97% in the low folate, replete, and high folate rats, respectively. For 5-FU, the TGIs were 46%, 49%, and 66%, and for doxorubicin the TGIs were 25%, 55%, and 61%, respectively. Using analysis of variance, the difference between the folate-deficient animals treated with cyclophosphamide and the folate-replete or supplemented rats was significant ( $P = .048$ ). The  $P$  values for 5-FU (.678) and doxorubicin (.442) were not significant. These results suggest that tumors in folate-deficient hosts are relatively resistant to cyclophosphamide, and that the efficacy of this drug almost can be doubled by correcting folate deficiency.

Because the efficacy studies described above indicated that folate status also might influence the toxicity of chemotherapeutic drugs, further specific investigation of drug toxicity was performed. Weanling Fischer 344 rats were divided into three groups of six animals each and maintained for 7 weeks on the low folate diet, a folate replete diet, or the latter diet supplemented with folic acid injections. Cyclophosphamide 62.5 mg/kg, 5-FU 75 mg/kg, or doxorubicin 7.5 mg/kg were injected on days 1, 5, and 9 (50, 55, and 59 days after beginning the special diets). The animals were weighed daily and observed for signs of toxicity: loss of grooming behavior, lethargy, and loss

Table 1. Folate Levels, as Measured by the *L casei* Method in MADB106 Mammary Tumors and Livers From Rats Maintained on Different Folate Intakes

Diet	Tumor*	Liver*
High folate	$0.280 \pm 0.044$	$6.2 \pm 2.8$
Folate replete	$0.091 \pm 0.074$	$3.2 \pm 2.2$
Low folate	$0.034 \pm 0.022$	$0.168 \pm 0.03$
Very low folate	$0.027 \pm 0.005$	$0.115 \pm 0.07$

\*µg/g wet weight.



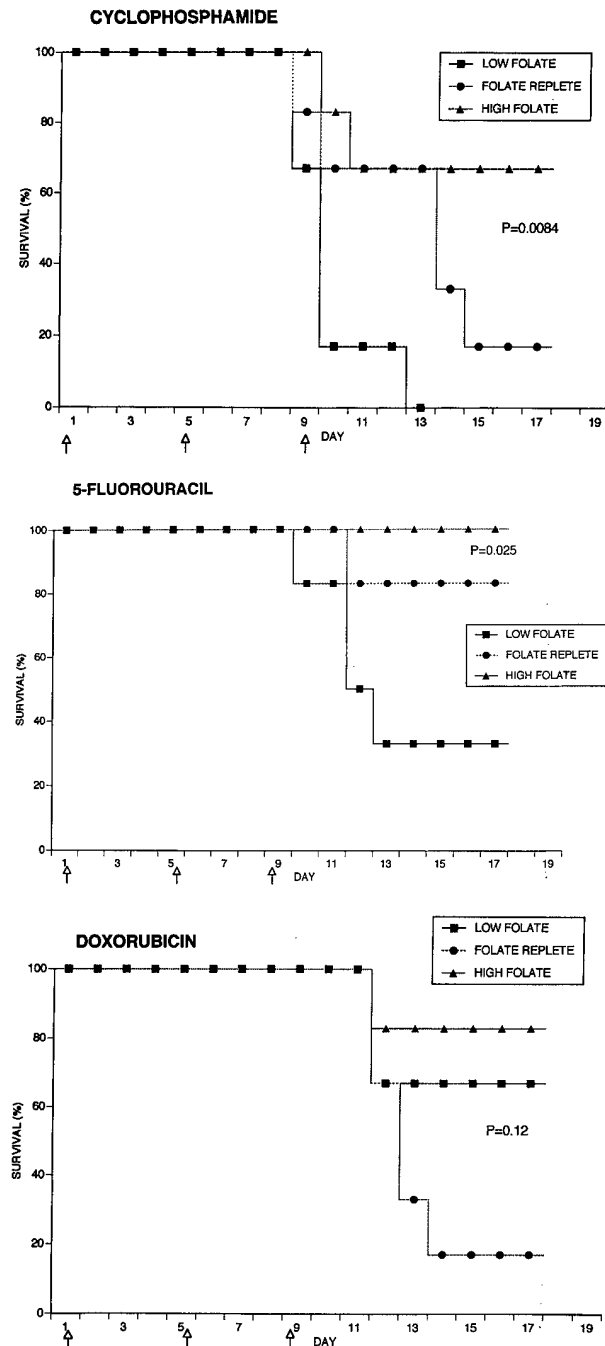
**Fig 3.** Inhibition of MADB106 mammary tumor growth by chemotherapeutic drugs in rats of varying folate status. (□), Regular diet; (▨), low folate; (▩), high folate. After 7 weeks on the indicated diet conditions, tumor was injected subcutaneously and six rats per group were treated with cyclophosphamide 50 mg/kg, 5-FU 50 mg/kg, doxorubicin 5 mg/kg, or vehicle alone. Two injections, 96 hours apart, were given when the tumors were palpable. Ninety-six hours after the second injection, tumor growth inhibitions were calculated. Using analysis of variance, the difference between the folate-deficient animals treated with cyclophosphamide and the folate-replete or supplemented rats was significant ( $P = .048$ ). The  $P$  values for 5-FU (.678) and doxorubicin (.442) were not significant.

of righting reflex. As shown in Fig 4, there were dramatic differences in survival in rats receiving cyclophosphamide or 5-FU related to folate status, with folate-supplemented animals doing better than folate replete animals, and both groups demonstrating better survival than folate-deficient rats. Thus, in the cyclophosphamide-treated group, all of the folate-deficient animals were dead by day 12, while the majority of the high folate animals survived until the end of the experiment, 1 week after the last injection of chemotherapy. Similarly, there were no deaths in the high folate animals treated with 5-FU, while two thirds of folate-deficient rats treated with this drug died by day 12. These differences were statistically significant ( $P = .0084$  for cyclophosphamide,  $P = .025$  for 5-FU) by the Log-Rank test. In contrast, doxorubicin-treated rats did not show a relationship between survival and folate status: the folate-supplemented rats did best, the folate-deficient group was intermediate, and the folate-replete group did the worst ( $P = .12$ ). Analysis of variance indicated no significant differences among the weights of the dietary groups treated with cyclophosphamide, but high folate rats lost less weight than other dietary groups after treatment with 5-FU ( $P = .0143$ ) and doxorubicin ( $P = .0067$ ) (data not shown). Measurements of liver folate levels in rats supplemented with folic acid indicated that chemotherapy with these three drugs did not change tissue levels of the vitamin compared with untreated control animals (data not shown).

#### DISCUSSION

The results of these experiments indicate that nutritional folate status influences the efficacy and toxicity of cancer chemotherapy in rats. The extent of this influence varied by chemotherapeutic agent. Cyclophosphamide, a bifunctional alkylating agent, was only half as effective at inhibiting the

growth of a rat mammary tumor and was much more toxic in folate-deficient rats. Although 5-FU and doxorubicin tended to be less effective at controlling tumor growth in folate-deficient rats, these effects were not statistically significant. The toxicity



**Fig 4.** Effect of folate status on the toxicity of cancer chemotherapy. Three groups of 18 rats each were maintained on the indicated folate diets. After 7 weeks, six animals from each group were treated with either cyclophosphamide 62.5 mg/kg (top), 5-FU 75 mg/kg (middle), or doxorubicin 7.5 mg/kg (bottom). Drugs were injected three times at 96-hour intervals (arrows). Survivals were significantly different for cyclophosphamide ( $P = .0084$ ) and 5-FU ( $P = .025$ ), but not for doxorubicin ( $P = .12$ ) by the Log-Rank test.

of 5-FU was significantly greater in folate-deficient rats than in folate replete or supplemented rats, while there was no clear effect of folate status on the toxicity of doxorubicin.

Considering the large number of patients who receive chemotherapy each year and the substantial number of these individuals who are likely to have a nutritional abnormality of folate metabolism, it is surprising that so little attention has been paid to the interactions of chemotherapeutic agents and folate. Parchure et al<sup>16</sup> found that high doses of folate potentiated the cytotoxicity of methotrexate, 5-FU, cytosine arabinoside, or mitomycin C against P388 lymphocytic leukemia in mice and prolonged survival, possibly by changes in purine and pyrimidine supplies. In contrast, folate supplementation (5 mg/day) of children with acute lymphoblastic leukemia in remission increased their tolerance of 6-mercaptopurine and raised a concern that folate supplements might interfere with therapy.<sup>17</sup> The ability of folate cofactors to enhance the cytotoxicity of 5-FU by forming a stable complex that inhibits thymidylate synthetase is well described,<sup>18</sup> and folate levels are known to affect the efficacy and toxicity of 5-FU. For example, Chéradame et al<sup>19</sup> have shown that the response of patients with head and neck cancer to 5-FU was correlated with tumoral reduced folate pools. The distribution of reduced folates was significantly higher for complete responders in comparison to patients with a partial or no response.<sup>19</sup> Recent studies in a dietary folic acid-depleted mouse model indicated that C3H mouse mammary adenocarcinomas were somewhat less responsive to 5-FU alone compared with folate replete animals.<sup>20</sup> Leucovorin administration 1 hour before 5-FU suppressed tumor growth 80% in folate-depleted mice. However, the administration of leucovorin at an improper time before 5-FU (12 hours) not only did not potentiate, but actually resulted in tumor growth stimulation.<sup>20</sup> Similarly, studies of the effects of folinic acid in 5-FU induced killing of human tumor cell lines in vitro found that the cell of origin, the dose, and the duration of exposure to folinic acid all influenced cytotoxicity.<sup>21</sup> The addition of very high doses of folic acid (125 mg/kg) increased the toxicity of 5-FU in mice.<sup>16</sup> In humans, stomatitis and diarrhea tend to be more frequent when folinic acid is added to 5-FU than with 5-FU alone and is the dose-limiting toxicity.<sup>22</sup> However, hematologic toxicity tends to be less with the combination of folinic acid/5-FU than with 5-FU alone.<sup>22</sup> These studies emphasize the complex interactions of folate status and chemotherapy.

A further example of this complexity is the interaction of folate status and antifols. In 1962, Potter and Briggs<sup>7</sup> reported that folate-deficient mice were extremely sensitive to amethopterin. More recently, this sensitivity was confirmed with 5,10-dideazatetrahydrofolic acid (DDATHF, Lometrexol). This agent interferes with de novo purine synthesis by inhibition of the two folate-dependent enzymes along that pathway, glycinamide ribonucleotide and aminoimidazole carboxamide transformylase. Mice fed a low folate diet for a short period became more than 1,000-fold more sensitive to the lethality of DDATHF than animals fed a standard laboratory diet.<sup>23</sup> Moderate folate supplementation (about 6 mg/kg/d orally) allowed complete suppression of C3H mammary adenocarcinoma without drug toxicity.<sup>23</sup> However, at high levels of folic acid intake (about 2,000 mg/kg/d orally), the antitumor activity of the drug was

completely blocked.<sup>23</sup> The applicability of these preclinical studies in rodents to humans is indicated by the observation that dietary folate supplementation of phase I patients has been reported to reduce DDATHF drug toxicity, allow further dose escalation, and preserve antitumor activity.<sup>24</sup> Thus, the effect of folate status on the efficacy and toxicity of chemotherapy appears to vary depending on the drug, the tumor cell type, and the folate level. In some circumstances, the correction of folate deficiency or additional supplementation may be beneficial, while in other circumstances, it may be detrimental.

Further complicating the issue is the fact that folate levels modulate tumor behavior independent of any effects on chemotherapy. The results presented here support older observations that dietary folate deficiency inhibits the growth of tumors.<sup>6,7</sup> We found that nutritional folate deficiency of moderate or marked degree significantly slowed rat mammary tumor growth compared with animals on a folate replete diet. However, additional supplementation with large amounts of folic acid daily did not significantly increase the rate of tumor growth compared with rats ingesting the folate replete diet alone. This observation is consistent with in vitro studies indicating that supplementation with folic acid above levels required for cell replication does not shorten the cell doubling time (reviewed in Branda<sup>25</sup>). Therefore, it appears that correction of folic acid deficiency will release the inhibition of tumor growth rate, but folate supplementation does not accelerate the rate.

Measurement of folate levels showed that tumor levels were lower than hepatic levels, but that the two levels were correlated. The folate status of the MADB106 mammary tumor recovered from rats on the folate replete diet,  $0.091 \pm 0.074$   $\mu\text{g/g}$  wet weight, was comparable to folate levels previously published for Walker carcinosarcoma 256 in rats ( $0.37 \pm 0.13$   $\mu\text{g/g}$  tissue) and for a variety of human cancers ( $0.05$  to  $0.5$   $\mu\text{g/g}$  of tumor).<sup>6,8</sup> Rosen and Nichol<sup>6</sup> concluded that the folic acid content of Walker carcinosarcoma 256 was less influenced than the liver by the dietary level of the vitamin because of the higher capacity of the liver to store folate. On the other hand, Gailani et al<sup>8</sup> found that in patients on a folate-deficient diet, the rate of folate depletion in the tumor tissue roughly paralleled the rate of depletion in liver and blood. More recently, Raghunathan et al<sup>26</sup> reported that accumulation of folate compounds was approximately simultaneous in plasma and mammary adenocarcinoma in mice, but delayed in liver. They found more active metabolism of folinic acid in the liver than in the tumor.<sup>26</sup> It appears, then, that generally there is a direct relationship between blood, hepatic, and tumor folate levels, but in some neoplastic tissues, the folate level may be low in the face of normal blood and adjacent tissue folate levels.<sup>27</sup>

Taken together, our studies and those of others indicate that folate status influences the efficacy and/or the toxicity of some, but not all chemotherapeutic drugs. It also seems likely that nutritional folate deficiency retards the growth of tumors. Therefore, correction of folate deficiency may be detrimental to the host if the tumor cannot be treated effectively. Alternatively, if effective therapy is available, our studies suggest that correction of folate deficiency can be beneficial. We found that cyclophosphamide was nearly twice as effective, with less toxicity, in folate replete compared with folate-deficient animals. Because slowly dividing cells are relatively resistant to

chemotherapy, the improved efficacy of cyclophosphamide in folate-supplemented animals may be secondary to increased proliferation compared with folate-deficient tumor cells. We recognize that rodents may not be an ideal model for the study of folate-chemotherapy interactions.<sup>28</sup> Therefore, extrapolation of these results to humans should await controlled clinical trials.

#### ACKNOWLEDGMENT

We thank Dr Takamaru Ashikaga for his assistance with the statistical analyses and Dr John Lunde for his review of the tumor histology.

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